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SOME PROBLEMS IN THE STUDY OF PHOTOSENSITIZATION[1]

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As an introduction to the problems with which this address deals we will note two phenomena with which the naturalist is familiar. The first of these is the synthesis of carbohydrates by chlorophyll under the action of sunlight. The second is fagopyrism, or buckwheat poisoning, in animals that have been sensitized to sunlight by eating buckwheat. It is known that black cattle that have eaten buckwheat suffer no ill effects from sunlight irradiation, that white cattle are sensitized by this food and show skin eruptions and lesions following sunlight irradiation, while spotted black and white cattle show the ill effects only in the non-pigmented areas.

These effects appear only if the sensitized animal is exposed to sunlight. If the animal is kept in a darkened stable no deleterious results of buckwheat feeding appear. Similar sensitization has been noted following the ingestion of other plants.

Von Tappeiner concluded that the active agent in photosensitization by buckwheat is an alcohol soluble fluorescent substance similar to chlorophyllan. Sheard, Caylor and Schlotthauer concluded that the fluorescence of chlorophyll, *per se,* as suggested by some investigators, is not

[1] Address of the president of the American Society of Naturalists, Indianapolis, December 30, 1937.

involved in the sensitization brought on by buckwheat. Grass is not toxic, but buckwheat is. They suggest that the photosensitizing substance may be phylloporphyrine.

It is evident that while the phenomenon just described may be regarded as an example of sensitization and reaction to light and that while it is a photosensitized reaction, it is clearly of a different character than the photosensitized reactions that constitute photosynthesis. The one thing that the two phenomena seem to have in common is that both are initiated by light.

In 1897–98 Raab, working under H. von Tappeiner's direction in testing the toxicity of the dye acridine on paramoecia, found that if very dilute acridine solutions containing paramoecia were exposed to light, the paramoecia were killed, while if cultures of this kind were kept in darkness, the paramoecia were unharmed. Investigations by von Tappeiner, Jodbauer and associates followed, and it was found possible to sensitize to visible light not only bacteria, protozoa and red blood corpuscles, but also enzymes, ferments, toxins, antitoxins and certain chemical substances. This sensitization to light von Tappeiner called photodynamic action. He defined this as sensitization by fluorescing stuffs.

Raab showed that the effective radiation was that absorbed by the acridine, and other work led to the conclusion that the effective region of the spectrum is that which is absorbed by the particular dye used.

H. F. Blum and his associates have shown convincingly that the mechanism of photodynamic action lies in activation of the dye molecule and finally oxidation of cell constituents.

We proceed, therefore, with the understanding that light of the wave-length absorbed by the dye in question activates the dye molecule and thus initiates a series of reactions ending in the oxidation of cell constituents. The phenomenon may thus be regarded correctly as following photosensitization.

With this brief introduction to the subject the speaker wishes to discuss certain results that have been obtained

on sea-urchin eggs irradiated in solutions of various dyes with sunlight. The work was undertaken by the speaker in a study of the experimental modification and control of cell division. The experimental part of the procedure has been done on the transparent eggs of the sea urchin *Lytechinus variegatus,* which are admirably suited for this work, at the Tortugas Laboratory of the Carnegie Institution of Washington.

About thirty dyes, one or more from each of the dye groups, have been tested. Of these only five or six will be discussed here. Various things, such as the dyes themselves, the concentration of the solutions used, the effect on fertilized and on unfertilized eggs, the duration of irradiation, the effect of temperature, etc., must be considered. The results to be presented were obtained with Neutral Red, Brilliant Green, Auramine O, Acridine, Eosin Y and Picric Acid, four of these being basic and two acid dyes. This distinction is made here in order that we may have in mind von Möllendorff's conclusion that basic dyes stain formed components in the cytoplasm of the living cell, while acid dyes form vacuoles or new components in the cell.

In the work with each dye it was necessary to find a concentration that was non-toxic to the experimental material in diffuse light or in darkness.

Early in the experiments it was found that uniformity of reaction was obtained only when the eggs were kept in suspension and stirred gently during the irradiation. By keeping them in motion it was possible to obtain uniformity of irradiation over the entire surface of the egg. If the eggs were allowed to settle to the bottom of the culture dish the effects were distinctly irregular. In tests with Neutral Red made to determine the degree of injury following irradiation of from 1 to 10 minutes it was found that the results gave a progressive series from no injury and normal division through irregularity of contour of the surface of the egg and modification of the mitotic figure, to slight cytolysis and finally to complete cytolysis.

Neutral Red, used in a concentration of 1:150,000, enters the eggs quickly, the eggs becoming stained brownish pink within three minutes after being placed in the solution. The brown color then fades, the eggs becoming distinctly pink. Within the next twelve to fifteen minutes the dye becomes concentrated in numerous Neutral Red granules, scattered throughout the cytoplasm, but always lying distinctly below the cortex of the egg. These statements apply to eggs in cultures kept in diffuse light. In cultures that have been irradiated with sunlight the reactions are quicker.

Neutral Red, in sea water solutions of a concentration of 1:150,000, used on uninseminated eggs and on eggs five minutes after insemination, was non-toxic in darkness, in diffuse light and on cultures exposed to sunlight for two minutes or less. Its photodynamic effect was increasingly evident in cultures sunned for two to eight minutes, and was destructive of all normal development in cultures that were irradiated beyond eight minutes. Following irradiation of twelve to twenty minutes there was complete cytolysis, the surface of the egg forming a mass of colorless blisters, while the center remained for a while as a core of Neutral Red. Ten minutes after the eggs were brought back from sunlight to diffuse light a most remarkable biological reaction began. The surface of the eggs broke rapidly into blisters, whose content was not watery but seemed similar to the cytoplasm of the egg. Sections of fixed preparations of these eggs show the plasma membrane distended by a flattened mass of hyaloplasm connected with the egg by a slender stalk. The term blister cytolysis describes this reaction accurately.

In eggs that were placed in the Neutral Red solution ten minutes after insemination, and therefore after the union of the germ nuclei, the results of short irradiation were less uniform. It was found that if the eggs were placed in the Neutral Red solutions after the nucleus had begun its processes of mitotic division, and sunned for two to eight minutes the nucleus and cytoplasm completed their

division, often with some modification, such as lagging and pycnosis of chromosomes, effects similar to the primary effect produced on cells by x-ray irradiation. It would be possible to make a phase-by-phase comparison of the photodynamic effect of Neutral Red on cell division with the phases obtained by Politzer and others after x-ray irradiation.

In the eggs given irradiation of shorter duration there was uniform behavior of the Neutral Red granules, these crowding in on the mitotic spindle as it formed, and then after the completion of division, returning to their former position slightly beneath the surface of the cell.

Neutral Red cultures exposed to sunlight, but protected by a red filter, developed perfectly. Cultures screened from ultra-violet light alone were killed by exposure to sunlight.

The result suggested heat cytolysis, and a study of the effect of increased temperature on these eggs was made. The effects of temperature will be discussed later.

Brilliant Green

This dye was used in concentrations of 1:50,000 to 1:1,600,000. The results are striking. This is not the place in which to consider details that can not be of general interest. It is sufficient to say that the treatments have revealed different degrees of cytolysis which if taken alone would merit a special classification. The least degree of cytolysis seems to lie in blister cytolysis, which may be of smaller or larger extent; beyond that disintegration of the egg may occur. The next degree of injury seems to result in extreme irregularity of the egg with the formation of lobes and lobules with or without the formation of blisters; a further degree of injury results in colorless cytolysis, the color of the dye fading and the egg breaking into innumerable fine, colorless blisters and finally so great an injury may result that the egg remains spherical, its surface coagulated so quickly that no superficial reaction is possible.

Colorless cytolysis is possibly to be interpreted as oxidation of the cellular constituents with reduction of the dye.

AURAMINE O

The results of staining with Auramine O are of a different nature than those following Neutral Red. With Auramine O the cytoplasm of the eggs stains a uniform lemon yellow. There are no Auramine O granules.

This dye was found to be exceedingly toxic. In concentrations of about 1: 5,000,000, development in diffuse light cultures was fairly normal, but was highly modified in those that were irradiated. It was strikingly evident that irradiation intensified the toxicity of this dye. The more striking features of the photodynamic effect of Neutral Red was the quick production of cytolysis. The primary effect of Auramine was not a visible reaction of the surface of the cell, but concerned internal activities.

If eggs, immediately after insemination, were placed in solutions of Auramine O that were too toxic, or that became too toxic upon irradiation, union of the germ nuclei did not take place, and the eggs retained a normal contour and underwent no further development.

If the processes of fertilization were completed before the eggs were placed in dilute Auramine O solutions and irradiated, the photodynamic effects were on the processes of mitotic division. The spindle was reduced in size, was usually multipolar, and division of the nucleus was not followed by division of the cytoplasm.

In the normal division of this sea urchin egg the division figure fills the cell, the radiations from the poles of the spindle extending to the surface of the egg. In the modified development just described, the spindle was reduced in size to about the length of the diameter of the nucleus, and in most cases normal radiations from the poles were completely suppressed. The result of treatment with Auramine O thus resembles the influence of narcotics on the cell.

On this highly modified spindle the chromosomes were divided, and because of multipolar spindles many partial nuclei, *i.e.,* nuclei containing only part of their chromosomal complement, appeared.

The second division followed the first after a short interval, the spindles formed being similar to those of the first division.

The multinucleate cells finally fragmented irregularly and cytolized.

Uninseminated eggs irradiated in Auramine O solutions and fertilized after removal to diffuse light showed the same reactions as eggs that were not irradiated until after insemination. The effect of increased temperature is of interest here. At 28° eggs in non-irradiated diffuse light cultures divided normally; those irradiated for ten minutes showed no cytolysis and gave nuclear division but no division of the cytoplasm; those irradiated for twenty minutes showed rare cytolysis but were otherwise like eggs of the ten-minute irradiations; while eggs inseminated at 28° and then allowed to come to just below 32° during a twenty-minute irradiation showed a good deal of fine blistering shortly after insemination, cytolysis increasing and disintegration following during the next twenty minutes.

Acridine

It is with interest that we turn to the effects of Acridine, as this is the dye that was used by Otto Raab. The least harmful solution used was a concentration of 1:640,000.

When the unfertilized egg is inseminated in a diffuse light culture a good fertilization membrane appears. Fertilization, however, is not completed, the union of the sperm and egg nuclei being blocked. Two nuclei, a large and a small, may be seen in most of the eggs. Irregular cleavage takes place in most of the eggs. A few remain undivided. Cytolysis does not occur.

When eggs are inseminated after twenty minutes' irradiation no fertilization membranes are separated, a large and a small nucleus may be seen in the egg, cleavage does

not take place, and although there is usually a slight roughening of the surface of the egg, cytolysis is rare.

In similar cultures allowed to rise to a temperature of 32° no fertilization membrane is formed, the cytoplasm becomes granular in appearance, the egg nucleus remains intact and becomes somewhat swollen, the egg and sperm nuclei fail to unite and about 10 per cent. of the eggs blister cytolize, these finally going to pieces.

These four basic dyes, Neutral Red, Brilliant Green, Auramine O and Acridine, have been selected for this discussion for the reason that each dye has a characteristically different effect on the egg. All these basic dyes enter the egg; some seem to stain only the hyaloplasm; some appear in the cytoplasm as a result of the affinity of these dyes for certain formed components of the cytoplasm.

The point to be emphasized is that in solutions of dyes not only is the surface of the cell in contact with the dye, but because the dye has entered and spread through the cytoplasm this also is subject to the direct influence of the dye.

Of the acid dyes Eosin Y was selected because of its strong fluorescence.

EOSIN Y

Experiments on fertilized eggs with dilute solutions of Eosin Y and of Fluorescein, even when the solutions in sea water were neutralized, were never satisfactory, for the reason that the eggs seemed protected by the fertilization membrane.

With unfertilized eggs, however, consistent results were obtained with solutions in sea water of a concentration of 1:150,000.

In diffuse light cultures a strong, well-separated fertilization membrane developed after insemination, and the eggs kept pace with those of the sea water control throughout cleavage and into the blastula stage.

In irradiated cultures inseminated after irradiation, although fertilization took place, the union of the nuclei

being observed, the fertilization membrane was not lifted from contact with the egg.

In all the irradiated cultures some blistering appeared within fifteen minutes after insemination. The striking feature, however, was pitting of the surface of the egg, followed by the formation of numerous pink vacuoles containing refringent granules. In all except the blistered eggs, which became spherical masses of small globules, mitotic figures appeared and the vacuolated eggs completed compact and irregular cleavages. At the end of five and one half hours, while both the sea water control and the diffuse light Eosin Y control had reached the normal swimming blastula stage, the irradiated cultures were increasingly vacuolated and cytolizing, depending on the duration of irradiation and the temperature to which the culture had been subjected.

Picric Acid

We will next consider another acid dye, Picric Acid, which while it has no photodynamic effect, is of help in the interpretation of some of the phenomena that have been considered. The results were unexpected. After trying out a 1:100,000 solution of Picric Acid in sea water, 1:50,000, 1:20,000 and 1:10,000 were successively tried, both in diffuse light and in irradiated cultures. It was found that the eggs in the irradiated cultures were distinctly ahead of both the control and the diffuse light cultures in rate of cleavage, due to an increase of 2° in temperature to which the eggs had been subjected during irradiation.

Subsequent experiments with concentrations of 1:8,000, 1:6,000, 1:4,000, 1:2,000 and 1:1,000 were then performed. It was not until the concentration of 1:2,000 was reached that the eggs were killed, the eggs remaining spherical, with an intact nucleus. In the weaker concentrations, 1:4,000 to 1:8,000, cell division was normal.

When eggs with a well-separated fertilization membrane were studied in a 1:1,000 diffuse light culture it was

found that fine blisters appeared immediately over the entire surface of the egg and within five minutes the egg became very yellow. In the irradiated culture the reaction was similar. To make the story complete it may be noted that when fertilized eggs are dropped into a saturated sea water solution of Picric Acid the surface of the egg is coagulated so quickly that it remains smooth.

These results with Picric Acid confirm the conclusions previously mentioned as to the protective nature of the fertilizing membrane against acid dyes and as to the effect of Brilliant Green solutions in coagulating the surface of the egg so quickly that no superficial reaction is possible.

DISCUSSION

Some comment on the technique of this work may be desirable. In every experiment a sea water control and a dark or a diffuse light control was kept. During irradiation the eggs were mechanically stirred and the temperature was controlled accurately with tubes of ice water. During the last summer's work in every experiment one irradiated culture was allowed to rise to a temperature of 32° and held there. Temperature control is essential. During a fifteen-minute irradiation of one of the darker solutions, of Nigrosine for example, if uncontrolled, the temperature may rise to 37°.

The first general conclusion concerning the photodynamic effect of these dyes is that in every instance irradiation with sunlight raised the effectiveness of dilute solutions to the level of results produced by strong solutions without irradiation. This observation simply confirms conclusions reached by other observers.

A second general conclusion is that every dye, if it has any effect at all, has a characteristic effect. The statement is made in this form in order to avoid the word "specific." The effect produced by one dye may be the same as that following the use of another. For example, Brilliant Cresyl Blue produces the same effect as does Neutral Red.

We have seen that Neutral Red, with enough irradiation to be destructive, produces complete blister cytolysis;

with less irradiation the result is lagging and even pycnosis of the chromosomes during cell division. The use of Auramine O results in failure of normal division of the cytosome, and syncytia are produced; that of Acridine, in failure of the union of the germ nuclei.

Mention was made of the fact that it was difficult to get any effect with certain acid dyes, if a well-developed fertilization membrane surrounded the egg. With an unfertilized egg a 1: 150,000 concentration of Eosin Y was more effective than was a 1: 12,500 solution on an egg protected by its well-separated fertilization membrane.

It will be remembered that not so many years ago the nature of the fertilization membrane was a subject of investigation. Is the fertilization membrane simply lifted from the surface of the egg at fertilization, or is it in the nature of a precipitation or a coagulation membrane formed during the fertilization reaction?

This difference in reaction to Eosin Y solution of fertilized and unfertilized eggs led to a simple experiment. Eggs were inseminated in a 1: 500,000 Heparin solution in sea water. A strong, normal fertilization membrane was formed. Other eggs from the same female were inseminated in 1: 100,000 solution of Heparin. The result was a barely distinguishable membrane, irregularly separated from the egg. The conclusion, which seems to be a by-product of this investigation, would seem to be that an outer egg membrane undergoes considerable modification during the fertilization reaction.

Another conclusion concerns the effect of temperature on the photodynamic reaction. In all the experiments at least three irradiated cultures were carried; one was irradiated for, let us say, ten minutes; another for twenty minutes, the temperature in these being held to the sea water temperature of that day, this at Tortugas running during July and August from 28° to 30.5°. The temperature of the third culture was allowed to rise to 32° and held there. Without exception the destructive effect of irradiation varied with duration of irradiation and with temperature.

When fertilized eggs were irradiated in sea water and the water allowed to come to a temperature of 32° their surface became highly irregular soon after their return to diffuse light and a lower temperature. Their injury was reversible; the eggs recovered and their development was normal. In the dye-solutions, however, the injury to the eggs was always greater in the cultures in which the temperature was allowed to rise to 32°. Irradiation in dye solution plus heat is more effective than irradiation alone.

Bělehrádek, in his recent book, in his discussion of the non-specific nature of heat-injury, points out that "among the various modifications of living systems producible by heat, there is none which might be said to be exclusively brought about by heat alone." The influence of increased temperature, within limits, in the acceleration of developmental process as well as chemical reaction is, however, too well known to need comment.

Von Tappeiner emphasized the importance of fluorescence in photodynamic action, and it was at first thought that the photodynamic effects were due to the fluoresced light. It was soon shown that light of this slightly longer wave-length was not effective in producing in the cell the changes under discussion.

Von Tappeiner recognized dyes of weak and strong fluorescence and pointed out that many dyes that are regarded as non-fluorescent show fluorescence. It is clear, as has been mentioned by several investigators, that photodynamic action is not proportional to the degree of fluorescence.

The possibility of the photodynamic effect by photocompounds of the irradiated dyes has been discussed by numerous investigators. Menke, working with the dye Fluorescein, has shown that a photocompound of this dye produced by prolonged irradiation with sunlight produces hemolysis in the dark to the same extent as Fluorescein in the light.

In the speaker's experience the results of the use of pre-irradiated dye solutions have been highly inconsistent. It is conceivable that irradiation insufficient to produce an effective concentration of the photocompound may have been responsible for some failure. Positive results following irradiation in these solutions suggests this as an explanation in some cases. It is conceivable that over-irradiation is responsible for other failures, but as to that there is no evidence.

It is a fact that the photodynamic reaction, as has been indicated, takes place quickly when eggs are irradiated in the dye solutions. It seems to be a fact that prolonged irradiation of a dye solution is necessary in order to produce an effective photocompound. Explanations suggest themselves, but they are highly speculative. Whether the formation of a photocompound takes place more rapidly if the dye solution is in contact with the oxidizable material, in this instance with the living substance, is a subject for further investigation.

Finally, it should be made clear that no attempt has been made yet to determine the threshold of any of these reactions or to control or to stop the reaction once it has been initiated. No exact measurements of intensity of irradiation have been made. Studies of that kind possibly could be made to best advantage with monochromatic light.

THE REPRODUCTION OF VIRUS PROTEINS[1,2]

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PROTOPLASM is the stuff of life. It has, for some time, been regarded as living matter in its simplest form. It has interested biologists for over a hundred years, and has been the subject of extensive physical and chemical studies. However, the present state of our knowledge of protoplasm is best attested to by the fact that we are gathered here in a symposium on the very nature of this material. We know much about protoplasm, yet we do not know what it really is. There is a vital difference between the grayish, translucent, slimy protoplasm which is the slime molds and the grayish, slimy material of certain protein gels, yet we do not know exactly what constitutes this vital difference. Biologists have, for many years, attempted to differentiate between the living and the non-living in protoplasm, without much success. According to Seifriz, many of the older ideas have been discarded and the discussion has settled down to the question as to whether a vital substance is concerned or whether protoplasm results from a certain combination of substances, themselves non-living. Most of the workers appear to feel that life results from a certain combination of a number of constituent parts, but that if a single vital substance is essential it is probably a prctein or protein complex. Since viruses have long been considered to be living organisms and since certain typical viruses have been isolated recently in the form of high molecular weight proteins, it is obvious that

[1] Read at a symposium of the American Society of Naturalists in joint session with the American Society of Zoologists, the Botanical Society of America and the Genetics Society of America. The American Association for the Advancement of Science, Indianapolis, Ind., December 30, 1937.

[2] The writer wishes to thank the many individuals who have contributed to the development and clarification of several of the points discussed in this paper.

a careful consideration of the nature of these virus proteins may throw some light on this question.

Viruses are of unusual interest in this connection, because with respect to size they bridge the gap between the living and the non-living. At the upper end of a scale (see Fig. 1) which includes entities ranging in size from the molecule of egg albumin, through viruses, up to the red blood cell, it may be seen that certain viruses such as vaccinia and psittacosis are larger than certain well-recognized organisms such as the pleuro-pneumonia organism. At the lower end of the scale certain viruses such as poliomyelitis and latent mosaic of potato are smaller than certain protein molecules. Viruses, therefore, form an unbroken line with respect to size from the molecules to the organisms, and it is impossible at present to draw a dividing line between the two. Now, let us examine the nature of these agents. I have said that certain viruses have been isolated in the form of high molecular weight proteins, and we know that others have been isolated in the form of heavy materials composed chiefly of protein. The ones so isolated include those of foot-and-mouth disease, the staphylococcus bacteriophage, latent mosaic of potato, tobacco ring spot, severe etch, cucumber mosaic, many strains of tobacco mosaic, the Shope papilloma, colibacteriophage, the Rous fowl sarcoma, vaccinia and psittacosis. Many of these have been isolated in such small amounts that it has been impossible to study them adequately, hence it is not known whether or not they really have the ordinary properties of molecules. It is possible that some are not molecules, but molecular aggregates or even small specialized organisms. Methods are now available for the preparation in adequate amounts of most of these entities, hence it is but a question of time until sufficient amounts for complete studies are prepared. However, certain of the virus proteins, notably tobacco mosaic, have been prepared in such large amounts that rather complete physical, chemical, biological and serological studies have been possible. These studies have enabled

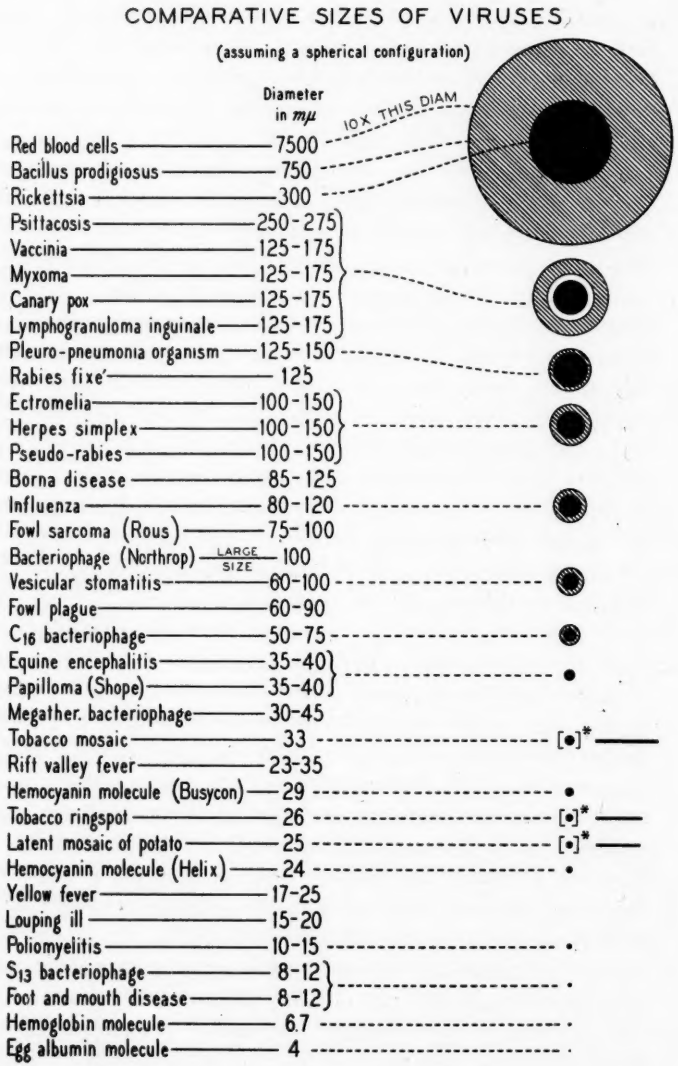

FIG. 1. A chart showing the relative sizes of several selected viruses including bacteriophages as compared to those of the red blood cell, *Bacillus prodigiosus*, rickettsia, pleuro-pneumonia organism and protein molecules.

a rather good yet still incomplete understanding of the probable nature of this virus protein. This afternoon I should like to discuss certain aspects of these studies which may be of value to us in understanding a bit about the manner in which protoplasm grows.

Tobacco mosaic virus was first isolated in the form of a high molecular weight crystalline protein early in 1935. The material first isolated was not homogeneous in all respects, but since that time it has been possible to prepare tobacco mosaic virus protein that is homogeneous with respect to its physical, chemical, biological and serological properties. I should like to dwell upon this last statement regarding the homogeneity of the virus protein only sufficiently to impress upon you that, according to all the tests that we have been able to devise, the protein is homogeneous in all respects, hence pure, hence is the virus. Among the physical tests that have been used is homogeneity with respect to rate of sedimentation, as measured in the Svedberg ultracentrifuge, and homogeneity with respect to electrochemical behavior, as measured in the electrophoresis cell. Chemical tests for homogeneity include fractionation of the protein by fractional crystallization and by centrifugation from solution under a variety of conditions, solubility studies, correlation of absorption spectrum with destruction spectrum and studies on partial inactivation. The biological tests include the determination of the virus activity of the various fractions of protein obtained by physical and chemical means. The serological experiments include precipitin, complement-fixation, and both *in vitro* and *in vivo* anaphylactic tests. In connection with these tests, you may recall that at the meetings a year ago the protein as then pre-

Three of the viruses have markedly asymmetrical configurations, and are represented by the broad lines drawn to the right of the circles. Although these lines are drawn to represent rods having a circular cross section and a volume equivalent to that of the sphere, they should be regarded merely as illustrating markedly asymmetric particles. The figures used in the chart have been arbitrarily selected from the data of Elford, McIntosh, Bauer, Schlesinger, Svedberg, and others.

pared showed a cross reaction with normal plant protein when tested by the very sensitive anaphylactic technique. It was suggested that this cross reaction might be due to the presence of a fraction of a per cent. of normal protein as an impurity. This has been found to be the case, and it is now possible to remove this impurity and to prepare protein that gives no cross reaction with other material even by the most sensitive serological tests. Thus, by all the tests that we have been able to devise, the tobacco mosaic virus protein appears to be homogeneous. There is always the possibility, of course, that the virus activity is not due to the high molecular weight protein but to an impurity that can not be detected by means now at our disposal. It should be recognized, however, that, by virtue of the experimental evidence for homogeneity already available, an impurity could hardly be other than a closely related high molecular weight protein, hence it would still follow that the virus is a protein. The possibility that the activity may be due to an impurity must always remain, regardless of the material under discussion. However, since there is no reason to believe that such a situation actually prevails in the case of the virus protein, we are unable, at the present time, to conclude other than that the high molecular weight protein under discussion is the virus. Now, these same tests that indicate that the virus protein is homogeneous may be used to demonstrate that it possesses the ordinary properties of molecules. As a matter of fact, the chemist, after a perusal of the physical and chemical properties of tobacco mosaic virus protein, has no difficulty whatsoever in coming to the conclusion that, despite its huge size, it has all the properties of a molecule and hence is a molecule.

However, we must remember that in addition to the properties which tobacco mosaic virus protein possesses as a molecule it also possesses virus activity, and the essence of virus activity is reproduction. It is this property of the virus proteins that may give us a lead in our consideration of the growth of protoplasm and in which

we are especially interested this afternoon. Let us examine the facts that we have at our disposal. The introduction of a few molecules or perhaps even one molecule of tobacco mosaic virus protein into the cell of a susceptible host is followed by the production within a very short time of millions of molecules of the kind introduced. The production is not confined to the one cell but spreads to adjoining cells, so that not one cell but millions of cells are involved in the production of the new molecules of virus protein. We know that the new virus protein is of the same kind as that originally introduced, because we can isolate it and compare its properties with the properties of the virus protein introduced. We say that under such conditions the virus protein grows or reproduces, for from a few molecules we secure millions of molecules. Now, what happens when we retain the virus protein in a test-tube or attempt to culture it on cell-free media or introduce it into dead cells or the cells of non-susceptible hosts? We know that nothing happens, the amount of the protein does not increase, reproduction does not occur. Viruses have been found to reproduce, therefore, only within the living cells of certain susceptible hosts. I have said that in such cells the introduction of one kind of molecules results in the production of millions of the same kind. This is true, but it is not the whole truth, for just as there is this remarkable tendency for the production of millions of molecules of the same kind, there is also a remarkable tendency for the production of a very few slightly different molecules. The subsequent reproduction of such slightly different molecules gives rise to what we call a new strain, and the whole phenomenon may be regarded as mutation. We recognize that such a change has occurred by the symptoms we observe and because it is possible by suitable technique to isolate such strains. Thus Jensen has been able to isolate from a pure strain of tobacco mosaic virus over 50 strains, each of which he considers to be different and distinctive, and Holmes has been able to isolate a strain of tobacco mosaic virus that is so

mild in its effect on the Turkish tobacco plant that it is difficult to determine its presence by mere observation. It might be argued that these various strains existed in the so-called pure strain as contaminants, but all the evidence obtained by Jensen, Kunkel, Holmes and McKinney indicates that the new strains arise from a single pure strain when reproducing within the plant cells. We may conclude, therefore, that tobacco mosaic virus protein may reproduce within the cells of certain hosts and that when it does it tends to mutate and to give rise to new strains. Now, this ability to reproduce and to mutate only when within the living cells of certain hosts is a property that we have not hitherto ascribed to ordinary molecules. We know, of course, certain autocatalytic reactions, such as the conversion of trypsinogen into trypsin *in vitro* on the addition of a little trypsin, in which a protein molecule appears to be reproducing itself, yet we also know that in such instances the precursor is chemically quite similar to the end product. The situation prevailing in the case of tobacco mosaic virus appears to be different, for here we have found no evidence for the existence of a precursor similar to the virus protein and the reaction has so far been carried out only within living cells. Despite the fact that the net result of virus reproduction may be regarded as similar to that of an autocatalytic reaction, there is at this time no precedent for regarding the reaction itself as a true autocatalytic reaction. I feel that it is better to admit our ignorance, as long as we are forced to the inside of a living cell to achieve a set of conditions in which to conduct our autocatalytic reaction. It is a fact that we do not know all that goes on within a living cell and, despite many attempts to duplicate it or the set of conditions prevailing within, we have been unsuccessful. We are forced to conclude, therefore, that, although tobacco mosaic virus protein has the ordinary properties of molecules, it also has the ability to reproduce and to mutate, properties not ordinarily ascribed to molecules, and hence that tobacco mosaic virus protein represents an entity unfamiliar to

us. Now, we may speculate concerning its nature and the manner in which it reproduces and mutates. I wish to impress upon you that this portion of the discussion is not based upon fact, but upon fancy.

Let us first consider whether tobacco mosaic virus differs from ordinary living organisms. The fact that it has proved impossible to cultivate tobacco mosaic virus in the absence of living cells is frequently cited as evidence that it differs in nature from ordinary living organisms. However, this property of obligate intracellular parasitism is not peculiar to viruses, for there are certain organisms that are known to be obligate parasites and to require the presence of living cells for reproduction. I do not feel, therefore, that our failure to cultivate viruses on synthetic media necessarily means that they differ from bacteria. The fact that tobacco mosaic virus protein may be crystallized has also been cited as evidence that it is not a living organism. Yet we know that crystallization is merely orientation and that orientation occurs in many living systems. Stretched muscle is orientated and gives an x-ray diffraction pattern that is characteristic of the crystalline state. At certain stages of cell division orientation occurs within the nucleus, for it becomes highly doubly refractive. I do not feel, therefore, that the fact that we can cause tobacco mosaic virus protein to line up in orderly fashion need necessarily mean that it is non-living. No evidence has been obtained that viruses have cell walls, and this might be used as a basis for establishing a difference were it not for the fact that the slime molds appear to grow without well-differentiated cell walls. The absence of a cell wall, therefore, need not necessarily mean that tobacco mosaic virus is non-living. Tobacco mosaic virus protein appears to possess a definite solubility, and it might be expected that this might be used as a distinguishing characteristic. However, if an organism were sufficiently small you might expect it to be governed by the ordinary laws of physics and chemistry. Furthermore, this property is relative, for if you move your point

of reference to the inside of a whirling centrifuge the virus protein loses this property and settles out as would any bacterial suspension. Another point that may be considered is respiration, for it is well known that nobody has been able to demonstrate that viruses respire, whereas it is usually possible to show that living things respire. However, even this point fails us, for we know that it is difficult, if not impossible, to demonstrate respiration in the cases of some living things, such as certain seeds or bacterial spores. It is obvious that we can take up the supposed differences and eliminate them one by one when considered individually. However, when we consider these differences collectively, one is not quite so certain, and I think you will agree with me that there is a tendency for a reasonable doubt as to whether viruses are really similar in all respects to organisms. This doubt becomes even stronger when we consider one additional factor, namely, the size of viruses. I have already pointed out that certain viruses are smaller than accepted protein molecules. We have always assumed that a certain degree of complexity was necessary for life. In its simplest form this complexity might conceivably be represented by the interplay of a very few, perhaps even two, molecules. It may appear very questionable whether or not the degree of complexity characteristic of living organisms can be woven into a single molecule. However, argument based on size alone is not valid over the whole range, for, if we consider vaccinia virus a molecule, we know that into an entity of the same size can be woven the degree of complexity under discussion, for the pleuro-pneumonia organism is just such an entity. Nevertheless, I think there must be a difference between entities, the properties of which may in one case be ascribed to the interplay of several molecules within a definite system and in the other case to a peculiar type of organization within one molecule. It may be possible to weave into one large molecule a structure that may be responsible for properties which are quite similar to the properties caused by the interac-

tion of several different molecules in a living system. I think that we may conclude, therefore, that, although it is impossible to decide whether viruses are different from ordinary organisms on the basis of any one of the differences, the collective picture strongly indicates that there is a difference between viruses and ordinary organisms as we know them.

It is interesting to speculate as to whether or not the fundamental difference is that in the virus proteins we have an example of the vital substance of protoplasm. The virus proteins isolated so far have been found to be nucleo-proteins having unusually high molecular weights. It may be possible that in this chemical combination of nucleic acid and high molecular weight protein we have sufficient organization within a single molecule to endow it with the life-like properties that characterize it. Such an entity might be regarded as the simplest type of organism, an organism from which all extraneous material has been removed and which has become so highly parasitic that it can reproduce only under very special conditions. Green has already pointed out that an intracellular microbe might be expected to undergo a loss of function due to the assumption of such function by the surrounding protoplasm. Such retrograde evolution he states might progress to the point where only a single molecule remains, and this molecule would be expected to possess unusual properties but would be functionally complete only when immersed in protoplasm. We would, therefore, have an entity possessing all the physical and chemical properties of a molecule and at the same time the potential properties of an organism, without it itself being a functionally complete organism. It is obvious that this description fits the virus proteins. It may be that there is something intermediate between such an entity and ordinary living organisms. Studies on some of the larger viruses such as vaccinia should settle this point. Whatever the outcome of these studies, it seems unlikely that they will alter our conception of the smaller viruses; hence we may proceed with our discussion of the virus proteins.

It is interesting to speculate on the manner in which tobacco mosaic virus protein brings about the production of more of the same kind of molecules when placed in contact with the protoplasm of certain cells. This mechanism is of tremendous importance, because it is possible that it or a similar one may be responsible for the production of all the specific protein molecules as well as other molecules within living cells. It may be considered to be the very basis of life. I think we may dismiss at once the mechanism by means of which cells divide and thus reproduce, for we are interested in a more fundamental mechanism, the one by means of which the cell grows until it is in a position for the second mechanism to operate. It seems unlikely to me that the basic mechanism is one in which the virus protein absorbs food, discards waste, grows and at some proper moment divides into two molecules after the manner of cells. A mechanism that I like to consider is one based on the surface forces which Dr. Langmuir has demonstrated so vividly in recent years and some of which he described on Tuesday. You all know how readily you can induce crystallization of substances by merely introducing a tiny crystal of the material into a saturated solution. The atoms come out of the solution and line up in a fashion that is predetermined by the pattern presented in the form of the introduced crystal. In some of the complex silicates such as $Na_2Fe_4Al_{14}B_6Si_{12}O_{59}$ you may have from five to ten different components present in the solution and yet they all arrange themselves according to the pattern that is presented to them. In some instances, this tendency to assume a definite arrangement is so strong that apparently it may occur without the addition of the pattern or crystal seed. In other instances, the arrangement or crystallization will not occur without the pattern first being present. Well do I remember the time at school when a certain compound was synthesized for the first time in this country. Despite repeated attempts under all kinds of conditions, the material could not be caused to crystallize. However, when a small amount of the mate-

rial in crystalline form, which had been obtained from abroad, was added, the compound synthesized in this country crystallized quite readily, and since then it has been possible to crystallize the material without knowingly adding seed crystals. We say that the laboratory has become "seeded," that small amounts of such crystals probably occur throughout the laboratory. Here, therefore, we have an example of an arrangement that was dependent upon the presence of a pattern and a demonstration that chemically undetectable amounts of material are probably sufficient to serve as a pattern. The forces responsible for this orientation may appear mysterious, yet they are definite and specific and they are real.

Now, let us consider first what may happen on the introduction of a virus protein molecule into a certain cell, and then consider whether or not we may interpret the growth of protoplasm in terms of this happening. It seems likely that in a living cell there exists a vast array of compounds ranging perhaps from salts and simple amino acids, through intermediate compounds such as carbohydrates and polypeptides, to complex compounds such as proteins. Consider whether the virus protein molecule is able through a characteristic structure and the surface forces that I have described to cause all the component parts to come from this storehouse of materials and to line up in orderly fashion alongside the pattern. If we may assume this degree of orientation, then I think we may assume that, inasmuch as the intact virus protein molecule represents a more stable form, there will be a mutual solvation of forces with the formation of the intact molecule which is then freed from its original pattern and ready to begin its own cycle. I have pictured this happening in a rather naïve manner for the sake of simplification. Actually, the synthesis of proteins probably results from a series of interrelated chemical reactions that are catalyzed by intracellular enzymes. In this connection it is interesting to consider that the final step to which I just referred may consist of chemical reactions catalyzed by the virus pro-

tein itself. It may be regarded as being similar to an ordinary enzymatic reaction except that in this case the intracellular reaction is catalyzed by the same kind of molecules that are introduced. It is obvious that for the successful operation of this mechanism there must be present in the protoplasm all the necessary component parts. If one or more is missing, the virus protein can not be built up. This serves to explain the specificity of viruses, for a virus would be expected to reproduce only in protoplasm containing the component parts of the virus molecule introduced. There is some evidence that tobacco mosaic virus protein is built from such component parts and not from the large proteins normally built up in such cells, for this virus protein reproduces itself in both Turkish tobacco and phlox plants, and it has been found by sensitive serological methods that the proteins normally existing in these plants are quite different. This is an indication that the virus protein is built up from smaller serologically inactive units that must be common to both plants.

Now, if we may assume this mechanism for the reproduction of the virus proteins, may we not assume a similar mechanism for the production of all the proteins that are synthesized within the cell, and hence for the growth of protoplasm? We know that some virus proteins reproduce quite rapidly and others more slowly, hence there must in the first instance be a tendency for the reaction to proceed rapidly and in the latter instance for it to proceed more slowly. Whether this tendency be real or result from the relative lack of a given component, if it exists, it would, together with the mechanism that has been discussed, be sufficient to explain not only the various factors involved in the reproduction of the virus proteins but also the orderly manner of the growth of protoplasm. There could be explained in this manner the reproduction of virus proteins, their reproduction only in certain living cells, the rapid rate of reproduction in some cases and the slow rate in others, the change or mutation of one strain into one or more other strains, the immunity from other

strains that results from infection with one strain and the failure of one virus to immunize against a different one. Furthermore, we see that the possibility of synthesizing virus proteins in the absence of living cells is not denied us, for such synthesis should occur provided the necessary components are provided and the proper conditions are achieved. If we are ever able to synthesize virus proteins in the absence of living cells, then we shall have gone a long way towards the synthesis of protoplasm.

I should perhaps remind you at this point that I am speaking of fancy and not of fact. I think it well occasionally to assemble all the facts at our disposal, to use them as a foundation and then to allow our thoughts and fancy to proceed and lead us where they may into the unknown, disregarding the absence of facts that so far we have been unable to wrest from nature. At worst, we may become imbued with erroneous impressions—and even they have occasionally been beneficial; at best, we may secure ideas that will enable us to establish as facts the very ones that gave rise to the ideas. Whatever may be your attitude with respect to this point, I think that you will agree with me when I say that the elucidation of the manner in which the virus proteins reproduce will serve to give us a clearer picture of the manner in which protoplasm grows. Because certain of the virus proteins are available in large amounts and lend themselves readily to experimentation, I think that we are now in excellent position to pursue with great expectation the problem of the reproduction of the virus proteins and eventually that more interesting problem concerning protoplasmic growth.

THE CHEMICAL NATURE OF THE PLASMA MEMBRANE AS REVEALED BY PERMEABILITY[1]

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PERMEABILITY studies afford not only facts on the penetration of substances into cells but also provide means to the formulation of working hypotheses of those plasma membranes which cover the protoplasmic surfaces of living cells and regulate their metabolism. It seems to be increasingly probable that Overton's (1899) idea that an emulsion of oily substances in water-soluble cytoplasmic substances makes up the plasma membrane. For many reasons it seems to be necessary to disregard much of the historical and even contemporary evidence of the presence of continuous lipoid plasma membranes, a concept often confused with Overton's ideas.

For the purposes of permeability we propose to consider all substances having no dissociation constants larger than 10^{-12} as non-electrolytes, and all substances with one or more dissociation constants larger than 10^{-2} as strong electrolytes. Different laws apply to the permeation of these classes of substances. Substances with dissociation constants between 10^{-2} and 10^{-12} may be classed as weak electrolytes. Weak electrolytes obey the laws governing either or both non-electrolytes or strong electrolytes according to the particular conditions and particular hypotheses which have been elaborated on the basis of simpler laws.

On the basis of this classification of penetrating solutes and their relation to one or more plasma membranes, we may distinguish three types of permeation in tissues. The simplest case is one in which a non-electrolyte diffuses

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through the external plasma membrane into the adjacent cytoplasm, following only slightly complicated laws of diffusion. Various adaptations of an essential Fick equation, $\frac{dn}{dt} = -kQ\ \frac{dc}{dx}$, using activities instead of concentrations, are practically satisfactory. We may derive a permeability constant which, although it neglects the unknown thickness of the plasma membrane, tells us the number of gram moles of solute penetrating in one second through one square centimeter for a concentration difference across the membrane in gram moles per cubic centimeter: $P = GM \cdot sec^{-1} \cdot cm^{-2}\ (GM \cdot cm^{-3})^{-1}$. This form is applicable at least in part to all solutes, including water. The second type of permeation is typified by the resorption of hexoses through the intestinal mucosa, and is general when an epithelium absorbs some substance (usually a non-electrolyte) through the plasma membrane at one side of the epithelium, alters it in the cytoplasm and allows the product to pass out of the cell through the plasma membranes at one or the other side of the epithelium. The steps are still obscure, but one may tentatively accept this general picture. Only activity gradients are here important as before.

The third type of permeation occurs only in the case of electrolytes, which pass through one or more membranes. In this case it may be supposed that a second important driving force—probably a potential gradient—is even more important than activity gradients. We may tentatively consider that the driving force is of the type $\frac{dc}{dx} \pm \frac{d\pi}{dx}$, where $\frac{d\pi}{dx}$ is the potential gradient. An equation of the type $\frac{dn}{dt} = -kQ\left(\frac{dc}{dx} \pm \frac{d\pi}{dx}\right)$ is the fundamental law. Potential differences exert relatively large driving forces, so that a potential difference of only one millivolt would be equivalent to a difference of activity of about 0.39 $GM \cdot \text{liter}^{-1}$ for K^+ in KCl. The sign of the p.d. of 10 mv. might well determine the direction of movement of the

ion. The potential differences may be due to the production of weak electrolytes, an aspect which will be discussed below. If the plasma membrane is a mosaic membrane, *i.e.*, one which permits oppositely charged ions at different points, then oppositely directed local p.ds would arise at these different points. The experimentally observed p.ds would be intermediate between the extreme values of the p.ds across the membrane.

For the study of plasma membranes it is desirable to treat both the relatively simple diffusion of the non-electrolytes and water and the more complicated process of accumulation of ions. The permeability with chemical transformation of hexoses, urea, etc., may be omitted in studying the chemical and physical structure of the plasma membranes.

Among non-electrolytes water assumes a unique rôle. Its penetrability greatly exceeds that of even the lower alcohols (Collander and Bärlund, 1933). Therefore water, the most polar of all solvents, passes most quickly through the plasma membrane. This very fact points to the presence of some material other than organic apolar solvents in the plasma membrane. In a loose sense we may speak about this constituent as containing water-soluble substances, including proteins, many of which are relatively water insoluble.

The work of Collander (1937) and his colleagues shows that the rates of entrance of representative non-electrolytes into vacuoles or cells of about twenty species of plants are roughly parallel to the partition coefficients between olive oil or ether and water. Improved agreement could possibly be obtained with other oils or mixtures. The linear relation between permeability and partition coefficient between oil and water is well shown by Fig. 1. But among the smaller polar molecules the penetrability exceeds that predicted by the rule just quoted, a fact usually attributed to the presence of aqueous phases in the plasma membrane in addition to oil phases. This conception, the lipoid filter mosaic, has been

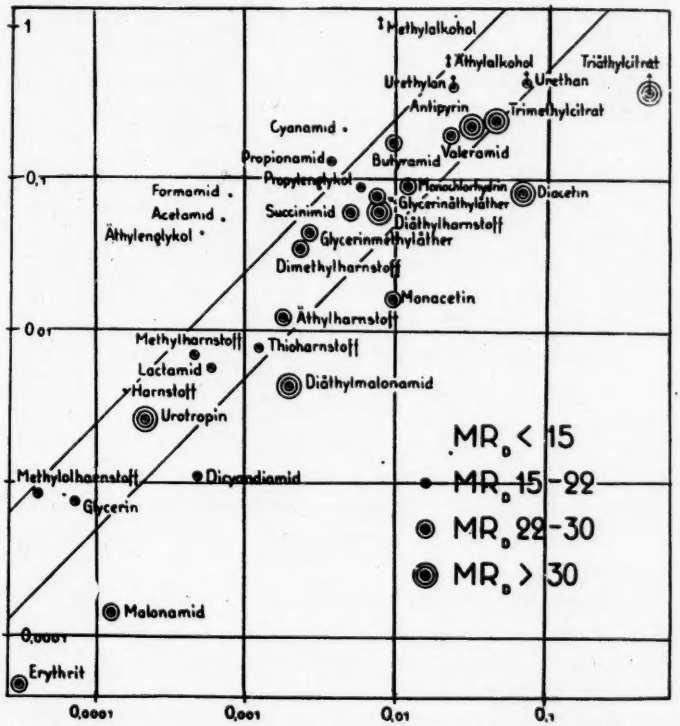

FIG. 1. The linear relation between permeability and partition coefficients between oil and water in the case of certain substances. Abscissas, partition coefficients (olive oil: water); ordinates, Permeability Constant, P. After Collander and Bärlund, 1933.

advanced by Collander (1937). This lipoid-filter membrane is much like Overton's membrane which contained lipoid dispersed in an aqueous layer as mentioned above. From the data given by Collander his conclusion as to the lipoid-filter plasma membrane may be accepted with certain possible modifications mentioned below.

From the experiments on non-electrolytes Collander and Bärlund (1933) have calculated the permeability of *Chara* by means of an equation $d = \frac{D \cdot n}{P}$ where d is thickness of the membrane, D the diffusion coefficient, n the

diffused substance in unit time, and P the permeability. Using the known area of external and vacuolar plasma membranes these membranes can be estimated to have an aggregate thickness of 3×10^{-5} cm, or 0.3 μ, provided that there is a continuous oily membrane. This is a microscopically perceptible thickness, and no such visible membrane has ordinarily been detected. A similar thickness has been postulated by Osterhout (1936). Collander has, however, pointed out that a lipoid-filter membrane might contain relatively small oil areas which could be supposed to have areas corresponding to the observed permeability.

The supposed existence of continuous films of oil may also be subjected to test by the following methods: Let us imagine that a thin layer of oil is present in or close to the surface of a cell, and that the stability of this film is due to the oil only. The interfacial tension, although low, is still positive. If a break in the plasma membrane occurs, usually due to experiment, the continuity of the film is broken.

In these experiments we can see microscopically the mixing of the aqueous matrix of the cytoplasm with the surrounding medium, and watch the granules and other particles diffuse out of the cell into the medium. If there had been present a continuous oil film, the existing interfacial tension of this supposed film would withdraw the oil from the break and the process would destroy the cell. But actually in many instances no such breakdown occurs, and one sees rather a healing by membrane formation over the opening. Therefore we can only conclude that something other than the oil film forms a new membrane, and the interfacial tensions, although opposing the reconstitution of the plasma membrane, must be not more than of secondary importance.

The experiments of Kopac and Chambers (1937) on the ingestion of minute oil drops by *Arbacia* eggs and other cells constitute a further test. An unprotected continuous oil film acting as a plasma membrane would fuse with an adpressed oil globule, so that the oil drop would spread

along the surface rather than pass into the cytoplasm as observed. Cells could not be covered with a naked oil film. In the case of vertebrate nerve axons Schmitt and Bear (1937) observed that a lipoid film exists in the surface. On both surfaces of this film proteins are arranged, and it is apparent that the stability of the thus-formed plasma membrane is primarily due to these layers of proteins. In more nearly normal cells it is rather doubtful whether such an oil layer is present at all, as shown by the same type of experiments. But even if *Arbacia* eggs have such a lipoid film with protective adsorbed proteins layers on both sides it is still doubtful that an applied oil drop would penetrate through the film at all or would penetrate without making contact with the lipoid. These facts seem to make the presence of continuous oil films improbable. With this background of the permeability to non-electrolytes and these correlated results, we are led to examine the evidence from accumulation of ions—the third type of permeability phenomena.

The accumulation of ions comprises a large array of conditions, in all of which ions are found in quite different concentrations or activities in the fluids on opposite sides of a plasma membrane. The disparity may commonly amount to such figures as fifty or one hundred fold; in extreme cases the disparity may reach several thousand fold. Although several mechanisms contribute to this disparity, like the well-known Donnan membrane equilibrium, still these figures transcend the combined possibilities of all these mechanisms, and other factors must contribute to the driving force by which ions penetrate. These inequalities of ion concentrations, giving the accumulation ratios, are due to simultaneous accumulation of *both* cations and anions, *viz.*, notably potassium and chloride, but also many other cations and ions. This simultaneous accumulation has been described most exhaustively by Steward (1937), Hoagland (1937) and their collaborators for plants. It is conceivable that amphoteric molecules might combine with both types of ions, and

this strongly suggests proteins or amino acids as the components active in ion penetration.

Several groups of phenomena are well known to be correlated with accumulation, notably oxidation in the sense that oxygen is consumed, the production of weak electrolytes and the presence of potential differences. Increase in size of cells and tissues is also related to these phenomena. These phenomena are generally recognized to maintain a dynamic equilibrium or a non-equilibrium state. This oxidative metabolism, or related reactions of living cells, provides a large source of energy. Accumulation requires osmotic work utilizing energy by means of some unknown mechanism, but it has been shown by Steward (1937) that in plant tissues only about one per cent. of the energy released is required for the known accumulation. In fact Steward supposes that protein metabolism might be the source of that faction of the energy utilized in accumulation. It seems probable that certain weak electrolytes produced in metabolism (possibly protein metabolism) produce the potential differences by diffusing out of the living cells, and that accumulation results from the counter diffusion of these weak electrolytes. The potential differences may be active in the process.

Two principal hypotheses of the accumulation process have been offered. The first hypothesis, enunciated by Osterhout (1936), assumes the presence of a layer of oil or other liquid "lipoid" substances completely enveloping the living cell. The evidence given makes it improbable that such a condition exists. It also appears that the experiments of Osterhout and co-workers are quite inadequate to prove the passage of salts by diffusion through such a non-aqueous solvent. Quantitative changes in ion concentration in the sap of coenocytes is only indirectly related to the permeability of either one of the two plasma membranes—vacuolar and external. Quantitative evidence on juice from cells, protoplasm, sap, cell walls and intercellular material or even data for isolated sap are likely to be misleading and are inconclusive. During

penetration of indophenols into the sap of *Valonia* coenocytes, it was shown by M. M. Brooks (1926) that sigmoid time curves of concentration appear showing a delay of indophenol into the sap. In the meantime the cytoplasm accumulates or stores indophenols. The resulting sigmoid curves have been demonstrated mathematically by such polyphase models (Fig. 2) (Brooks and Brooks, 1932).

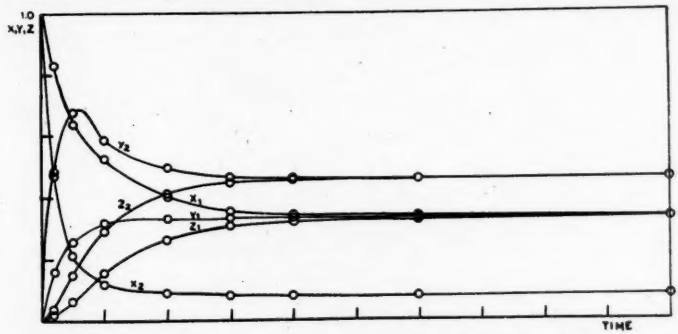

FIG. 2. The time curves of the concentrations of a single diffusing substance in the external medium (x), cytoplasm (y) and vacuole (z) of a model cell, without accumulation. The subscripts 1 and 2 apply to two different conditions of solubility of the substance.

Brooks (1935) has shown that rubidium and radioactive potassium ions (1937) are stored in the (separated) cytoplasm of *Valonia* and *Nitella* coenocytes during accumulation of strong electrolytes, and the ensuing concentration curves take a sigmoid form even more pronounced than the curves for indophenols (Fig. 3).

Former work has been based upon the differences in concentrations of similar electrolytes in the sap and the external solution without reference to the protoplasmic layer between them. In some of the cases studied, the diffusing material reaches the sap through several phases such as the external and vacuolar membranes and cytoplasm following an essentially exponential concentration-time curve. But it is difficult to establish that data on such complex cases are true for the simple component processes of diffusion and permeation. Time curves obtained by

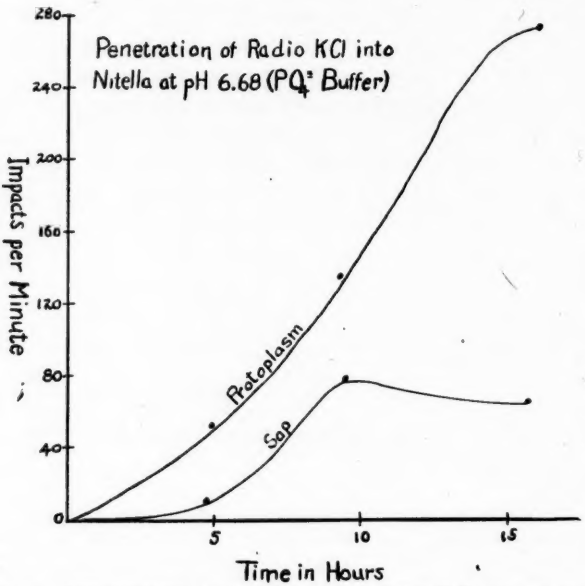

FIG. 3. Concentrations (ordinates) of radioactive KCl into the sap and protoplasm of *Nitella*. Abscissas, time.

study of the cytoplasm separated from the medium by only one plasma membrane are of primary importance. The development of this aspect of permeability offers an opportunity for new and valid data—a new clue to the nature of the plasma membranes.

Much of the earlier work yields quantitative data on the combined sap and protoplasm such as would be obtained in crushing tissues and extracting the juices. Since it is impossible in these cases to separate the data of each constituent, *i.e.*, sap and protoplasm, and in view of the large differences in concentrations found in experiments on the penetration of radioactive K (Brooks, 1938) in which the sap and the protoplasm have been separated and the K has been individually measured, these experiments, therefore, lose much of their significance. The conclusion that ion exchange is excluded and molecular diffusion favored by such data as the magnitude of changes in the sap alone

of coenocytes like *Valonia,* in which the ions have passed through the cytoplasm and the two membranes after an arbitrary lapse of time, must be modified.

In 1929 I suggested that any weak electrolyte might be responsible for accumulation, and I cited carbon dioxide as one of the weak electrolytes instrumental in accumulation. It is possible that carbon dioxide is not of general significance, but the presence of other unknown weak or strong electrolytes gives a sound basis for the accumulation of ions according to this hypothesis. One of the principal objections against this mosaic idea is based upon the idea that an excess of hydrogen ions on the side of accumulation is necessary. This presupposes that the weak electrolytes are acids, but it is quite possible that weak bases are present and function under at least some pH ratios. It is also true that the work of Hoagland and co-workers (see Steward, 1937), and of Jacques and Osterhout (1935), which report accumulation against an H-ion activity gradient are based on either an observed pH of an extract of cells or of the *sap* of *Nitella* rather than the pH of the *cytoplasm.* The amassed data on the pH of the cytoplasm of accumulating cells are only fragmentary. No clear conclusion can be reached until a large mass of data can be obtained.

In formulating the mosaic-ion exchange hypothesis of accumulation, I adapted an idea suggested by Höber and Höber (1928). This is the mosaic membrane, and this mosaic membrane should be thought of as a combination of lipoid and a mosaic made up of different portions which are at least partly protein in composition. The amphoteric protein-containing mosaic membrane must be thin, so as to admit cations at some points and anions at other points, corresponding to the acid and basic side chains of proteins or other constituents.

I have already described at considerable length the action of mosaic membranes by ion exchange (Fig. 4) (Brooks, 1929). This supposes that ions of the weak electrolytes produced by metabolism are exchanged for

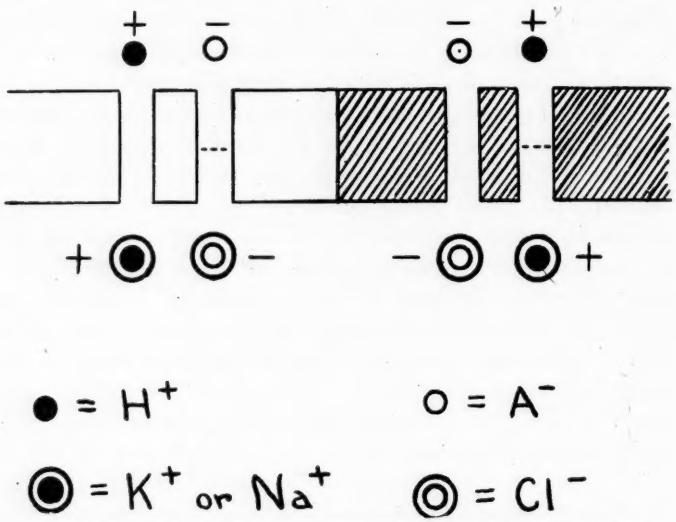

FIG. 4. Diagram of a mosaic membrane in radial section for KCl, NaCl and weak acid, HA. Absence or impermeability of pores indicated by dotted lines. Anion permeable area shaded.

ions of strong electrolytes from the external medium. The cations permeate through the cation-permeable points, and the anions at the anion-permeable points. This idea has been criticized by Briggs (1930) who postulated the simultaneous exosmosis of an oppositely charged ion, which would obviate the exchange of similarly charged ions. However, I have shown that both the processes must occur, *i.e.*, both that postulated by Briggs (1930) as negating accumulation and the ion exchange due to the presence of cation and anion permeable points which permit accumulation. The quantitative importance of these processes depends upon ill-defined factors. One of these factors is the thickness of the plasma membrane as compared with the distance between oppositely permeable areas. A thickness of about 10^{-7} to 10^{-6} cm is suitable for ion-exchange accumulation by my hypothesis, and agrees with the work of Fricke (1935), Cole (1937), McClendon (1926) and others on the electrical properties of the plasma membrane. Osterhout (1936) has adopted

Briggs's position exclusively, and supported this by erroneously citing an article by Söllner (1932). Since Söllner unqualifiedly supports the mosaic idea, we may discount this criticism.

Because the mosaic theory accounts for ion accumulation we may ask whether the work of other workers in discrete lines of investigation will provide a physical basis for this scheme. The work of Gorter (1937), Rideal (1937), Schulman (1937), Clark (1937), Langmuir (1937) and others on protein monolayers, which are supported by x-ray diffraction spectrography of Astbury (1936) and co-workers, and the mathematical considerations of Wrinch (1937) provides a tentative model of a plasma membrane. Among the most stable monolayers are those made by forming a protein monolayer on a water-air surface, under which were introduced such substances as cholesterol, lipoids, digallyl and in general water-insoluble substances. These often chelate molecules have been found to penetrate into the monolayer coupling with proteins at two or more points. This monolayer is remarkably like a lipoid-filter membrane, and the side chains of the proteins combine with cations and anions. The points of combination are more or less separated and would act as cation and anion permeable points.

The forces playing in the monolayer are remarkably like those observed in living cells. The tensions may be low, and it has been shown that compression lowers such a tension—that is, increases the pressure in the two-dimensional space. What knowledge we have shows that the tension decreases with decreased stretching to low values as shown by Cole (1937) for sea urchin eggs. We are also intrigued by the anisotropy of protein molecules, a property which suggests such forms as those of erythrocytes.

At about this point we are faced with the difficulty that while these beautifully adaptable monolayers form on a water-air interface, there are no interfaces between the freshly exposed cytoplasm and the aqueous external medium upon which the monolayers can form. The proteins are not constrained, with polar and apolar sides in the

water and air phases. But a membrane may form on the meeting of the cytoplasm and the external medium. It has been suggested by Heilbrunn (1928) that a surface precipitation reaction occurs, and certain striking analogies between this reaction and blood clotting are pointed out.

At present it is difficult to evaluate the significance of many of the details suggested by Heilbrunn. But the important part played by alkaline earth metals, notably calcium, and the function of proteins seem to be fundamental. In at least the case of the sea urchin eggs, *Arbacia,* the part played by the pigment granules which disappear during the diffusion of the exovate at the margins are necessary to the surface precipitation reaction. In view of the probable nature of the pigment granules as mitochondria, it is probable that at least some oil-soluble substances are liberated. It appears to be most probable that in the diffusing layers of cytoplasm a reaction appears as a result of cell disruption, and precipitation of protein molecules, cholesterol, lipoids and similar substances, together with a minimum amount of Ca^{+} ion occurs. This precipitation process physically resembles many such reactions as Traube's cell (cupric ferrocyanide), silicate and gelatin-tannic acid cells, and may comprise protein molecules, sterols, etc., and calcium monolayers as studied by Rideal (1937) and Schulman (1937). The presence of calcium is in consonance with the tendency of calcium and magnesium to favor small spreading of protein films, while potassium and sodium favor great spreading. This has been pointed out by Gorter (1937) in connection with physiological ion antagonism, which is widely observed.

Other aspects of the physiological effect of calcium reenforce this conception. Solid masses of cytoplasm occur when large concentrations of Ca^{++} occur in this cytoplasm. These have been observed by many workers. The vacuolation, *i.e.,* formation of some type of vacuolar plasma membranes, has been considered by Heilbrunn to be chemically equivalent to the surface precipitation reaction.

This general unity of plasma membrane formation is striking, even although it is well known that differences in permeability between vacuolar and external plasma membranes exist. The precipitation membrane, which is comparable with the plasma membrane, is produced by diffusion and reaction of common ingredients of the protoplasm. In the same way protoplasm enlarges or shrinks its surrounding plasma membrane by adding or removing components with changing area of surface. This enlargement of the erythrocyte plasma membrane during hemolysis has been deduced from electrical measurements by Curtis (1936).

The nature and arrangement of protein molecules in the plasma membrane is made most doubtful, since there is present not a monolayer *on* an interface, but a precipitation film found *in* an aqueous solution. There has been much discussion of spherical molecules whose molecular weight is about 34,000, or multiples of them, of extended chains or discs of four such units have been tentatively suggested. In the formation of protein films two types of side chains exert forces—the polar side chains by coordinated hydrogen linkages, the apolar side chains by van der Waals's forces. Addition of sterols and lipoids and of divalent cations must lead to chemical linkage, and changes in the proportions of sterols to lipoids and of calcium and magnesium to sodium and potassium are well known to affect the permeability of living cells.

Some possible arrangements may be examined. Regarding dimensions, we may only hazard the guess that a plasma membrane so formed might have a thickness of two side chains plus the central "backbone" of the protein amounting to about 1.5×10^{-7} cm, or some low multiple of this. As an alternative we may consider that the proteins, not being arrayed on a water-air interface, might lie on edge—adjacent proteins linked by alternate faces—polar and apolar side chains with interlarded discs of several kinds of oil-soluble molecules (Fig. 5). This would give about 40×10^{-8} cm. With any of these arrangements we may suppose that ion-selective points lie scattered at

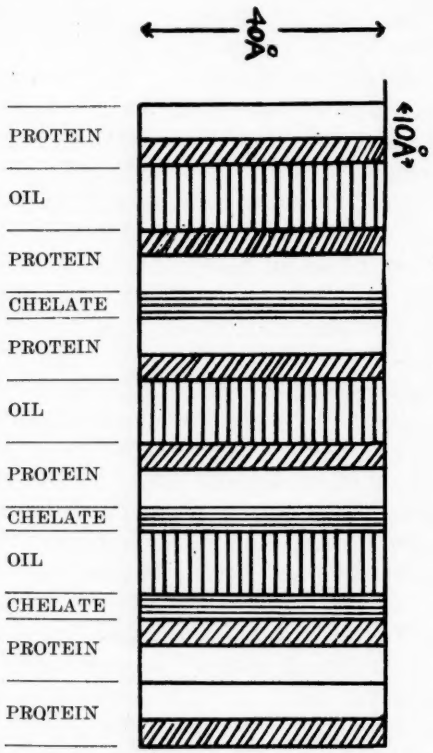

FIG. 5. Diagram of a mosaic membrane, containing protein molecules and other classes of substances. Various types of bonds are illustrated.

distances of from 3 or 4 up to 40×10^{-8} cm apart. If one knew these distances and the thickness, one might calculate the relative importance of ion-exchange through the membrane on one hand, and compensation of passage by a cation by the simultaneous passage of an anion through the membrane on the other hand. We may imagine a cation changing places with the H-ion of a polar side chain and progress through the membrane by repeating this process on successive side chains. Similar considerations apply to anions. The direction of this movement results from the counter gradient of similarly charged ions of weak electrolytes. If any significance accrues to the

process of ion-exchange—exchange of different cations or of different anions—we must support this conception of the mosaic plasma membrane.

The stability of the plasma membrane seems to be derived from the alternation of two or three different classes of substances in the precipitated film. But the presence of relatively large areas of oil, demanded by workers on the permeation of non-electrolytes, would make such a membrane unstabile or mechanically weak. Can non-electrolytes such as urea, substituted ureas, alcohols, amides, tri-ethyl citrate and a host of others pass through such a small area of but a few Ångström units as our theory requires? It seems quite possible that all these substances can "dissolve" in the "oil" of some component portion of such a plasma membrane, that is, these substances can pass between molecules or temporarily displace the molecules of the membrane.

Because of the fact that at least two independent paths exist through the plasma membrane, one "aqueous" and one "oily," we can understand the independent penetrabilities of different classes of substances. A host of baffling experiments may yield more easily to the use of the new concept of the plasma membrane. Regardless of the validity of the details of arrangement and bonding of molecules in the membrane, we may hope that a molecular conception, which can dispense with continuous oil films, can account for the known permeability of individual or several independent plasma membranes and may open up the province of more exact study of the structure of plasma membranes.

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THE PHYSICAL STATE OF PROTOPLASM WITH SPECIAL REFERENCE TO ITS SURFACE[1,2]

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THERE are two important conditioning factors in the investigation of protoplasm. One is the microscopic dimensions in which protoplasm universally exists (even in so-called giant cells the thickness of the protoplasmic layer is measurable in terms of a few microns only). The other is the inseparability of at least three components of protoplasm: the nucleus, the cytoplasm and the protoplasmic surface film.

The nucleus has not been universally regarded as essential, since, for example, enucleated bodies of protoplasm are known to exhibit properties of life, albeit somewhat restricted. However, there is evidence that the nucleus exerts a continuous influence upon the cytoplasm so that the cytoplasm without its nucleus can not be regarded as similar to nucleated cytoplasm. An example of this is the experiment (Chambers and Fell, 1931) on the frequently occurring binucleated fibrocytes in tissue culture. If the nucleus of a uninucleated cell is punctured with a microneedle the cell invariably undergoes degeneration and death. On the other hand, in a binucleated fibrocyte, the degenerative changes initiated by puncturing one of the two nuclei are stopped and reversed by the presence of the other nucleus.

We must also consider the presence of the nucleus as influential in the repair of injured protoplasmic surfaces.

[1] Read at a symposium of the American Society of Naturalists in joint session with the American Society of Zoologists, the Botanical Society of America and the Genetics Society of America. The American Association for the Advancement of Science, Indianapolis, Ind., December 30, 1937.

[2] All photographs of the figures in this paper are taken from a motion picture film entitled "Experimental Studies on the Protoplasmic Surface of Echinoderm Eggs," prepared by Mr. C. G. Grand in my laboratory.

If an amoeba is torn with microneedles the exposed cytoplasm starts to flow out and undergo degeneration. If the nucleus is at a distance from the tear, repair takes place by the formation of a film-like membrane running in a more or less straight line across the gap and excluding the outflowing, disintegrating cytoplasm. However, if the nucleus happens to be close to the surface which is being torn, the nucleus may be included in the outflowing cytoplasm, and the repairing surface, evidently influenced by the presence of the nucleus, tends to run in a sweeping curve around the cytoplasm so as to enclose the nucleus.

The interrelationship between nucleus, cytoplasm and protoplasmic surface must be borne in mind when conclusions are being drawn from experiments involving their isolation.

THE NUCLEUS

The normal, interkinetic nucleus, generally, is an easily deformable, almost fluid body. The lack of a visible internal structure may be due, in many cases, to a lack of sufficient optical refractivity. In the nucleus of the frog's egg there have been recently described not only definite structural components, *e.g.*, chromosomes, but also regional differences in consistency of the nuclear matrix (Duryee, 1937).

A pronouncedly fluid state of the nuclear interior can be demonstrated even in the much-branched nuclei of the silk-gland cells of caterpillars. An aqueous solution of a dye injected into the nucleus of one of these cells spreads rapidly into all its ramifications throughout the cell. On the other hand, the solution, when injected into the cytoplasm, spreads with difficulty, and dissection of the cell shows the cytoplasm to be highly viscid. When the cytoplasm is torn the exposed lobes of the nucleus become confluent, indicating that at least one of the conditioning factors for the peculiar shape of the nucleus is the unequal pressure upon it by the surrounding viscid cytoplasm (Chambers, 1938).

The nuclear membrane of nuclei mechanically isolated in physiological media has a relatively tough texture. In living cells its physical state is more difficult to determine. The nucleus of the fresh-water amoeba is a tough solid which, after being stretched with needles within the amoeba, will retain its distorted shape for several minutes. On the other hand, the germinal vesicle of Echinoderm eggs is a fluid sphere bounded by a film which, as long as it is retained as an intact envelope, reacts to micromanipulation as if it were a liquid insoluble in the two media which it separates, *i.e.*, the nuclear interior and the surrounding cytoplasm.

Some experiments have been performed on the permeability of the nuclear membrane. It is known that the nucleus swells together with the egg in hypotonic and shrinks in hypertonic solutions. However, evidence from microinjection experiments indicates that the permeability of the nucleus is of quite a different order than that of the cell surface (Monné, 1935). The distribution of a dye between the nucleus and the cytoplasm seems to be in accordance with the tendency of the dye to accumulate

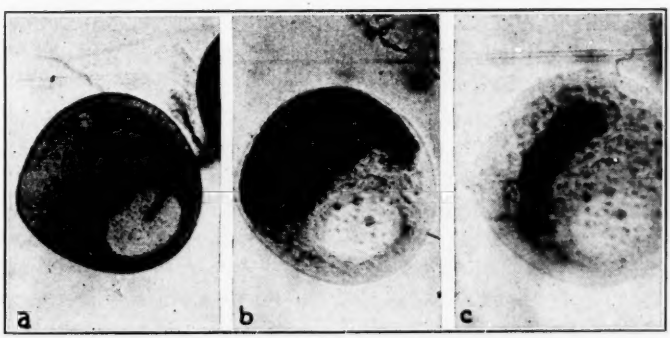


FIG. 1. a, b, c. Three successive views (within 8 seconds) of immature starfish egg after momentary puncture of nucleus in hanging drop of artificial sea-water. a, Egg prior to puncture with microneedle coming up from beneath. b, Spreading cytolysis three seconds after puncture. Note that cytolysis tends to be superficial with elevation of vitelline membrane. c, Cytolysis almost completed with debris filling distended vitelline membrane.

in whichever of two media it is more soluble, the two media being separated by a membrane to which the dye is freely permeable. For example, phenol red, a salt of an acid dye, injected into the cytoplasm will accumulate in the more alkaline nucleus, while neutral red, a salt of a basic dye, will not diffuse into the nucleus and, if injected into the nucleus, quickly diffuses out and accumulates in the more acid cytoplasm (Chambers and Pollack, 1927; Chambers, Pollack and Hiller, 1927).

At the same time the nuclear membrane must be keeping apart two strongly interacting substances, since a slight tear of the nuclear membrane with a microneedle is followed by a more or less extensive cytolysis of the surrounding cytoplasm, Fig. 1. Of significance in this regard is the marked susceptibility of eggs to mechanical injury during the period when the nuclear membrane normally breaks down prior to mitosis (Chambers, 1921).

THE CYTOPLASM

It is well recognized that the cytoplasm may exhibit marked variations of solidity and fluidity with definite evidences of reversible gelation and solation.

A question of interest is whether protoplasm when in a fluid state still has the properties of an elastic gel. The presence of Brownian movement in the heterogeneous cytoplasm does not necessarily indicate a liquid state, since granules in regions which are known to be gelated may still exhibit the movement. The existence of elastic properties within the cytoplasm may be determined by stretching an egg with two needles and observing the effect on the general trend of the Brownian movement. This experiment was done on the mature, unfertilized egg of the Arbacia and time-lapse motion pictures were taken to show the movement of the cytoplasmic granules. Irrespective of whether the egg was stretched or unstretched the granules still showed the same random movement, suggesting that the cytoplasm of the unfertilized egg is a liquid and not an elastic gel.

There are two fairly objective and delicate methods for determining the presence of localized and oriented variations in consistency of the cytoplasm. One is the direction taken by growing ice-crystals in supercooled cells (Chambers and Hale, 1932) and the other the form taken by microinjected oils which acquire a very low interfacial tension against cytoplasm. Freezing was induced within the supercooled cells by inserting an ice-tipped micropipette into the interior of the protoplasm. In the supercooled, fluid cytoplasm of the amoeba the ice formed as fine, feathery crystals, which grew in all directions around the vacuoles and the nucleus. In a muscle fiber the ice took the shape of slender columns extending lengthwise along the more fluid interstices between the denser myofibrillae.

Paraffin oil, injected into the mature sea-urchin egg, acquires a very low interfacial tension, so that the oil can be pulled into long slender strands. If this is done in the more fluid regions the strands break into isolated, spherical droplets. If the oil is injected into a gelated region the drop is not spherical. For example, an oil-drop, injected among the astral radiations, is conical, with the narrow end directed toward the center of the aster. This confirms previous conclusions from microdissection experiments (Chambers, 1917) that the aster consists of radially arranged columns of gelated material, the viscosity of which diminishes toward the periphery of the egg.

The miscibility of protoplasm with water is an important consideration. In general, cells which normally exist in a saline environment swell appreciably in hypotonic and shrink in hypertonic solutions, indicating that water can readily pass into and out of the egg. When water is injected into an Arbacia egg immersed in normal sea-water, the egg begins to swell, but within a few seconds returns to its original size, indicating the rapidity with which water diffuses out. The injection must be performed gradually in order to prevent cytolytic effects. The water miscibility of protoplasm is definitely conditioned by the

salts dissolved in the water. For example, an egg can be injected more easily and be made to swell to a larger size without evidence of cytolysis if the injected fluid contains potassium chloride than if it were distilled water only. With distilled water alone, the contingency of success upon the slowness of its introduction into the egg is presumably due to a dilution of the water with the salts in the egg.

Apparently, therefore, the protoplasm of these eggs has a high degree of water-miscibility but possesses a surface which, although readily permeable to water, is sufficiently insoluble to keep the protoplasm intact in an aqueous environment.

THE PROTOPLASMIC SURFACE

A better realization of what is meant by the protoplasmic surface can be had by a diagrammatic sketch, Fig. 2, of the cortex of the Echinoderm egg, based on conclu-

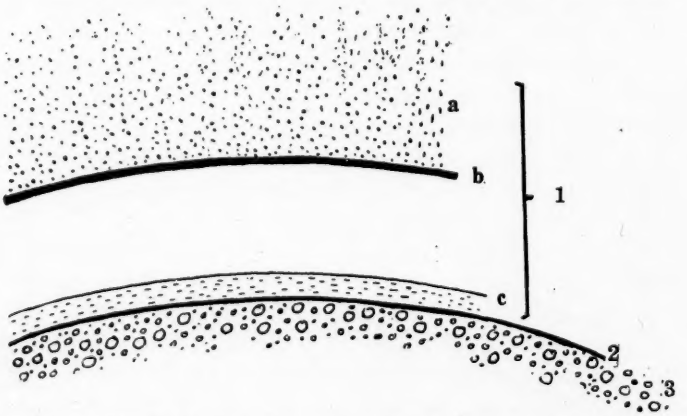


FIG. 2. Diagram to show three components of cell cortex of a sea-urchin egg. (1) Extraneous investments: a, jelly; b, fertilization membrane; c, "hyaline plasma layer or membrane." (a and c can be removed chemically. b can be removed mechanically.) (2) Protoplasmic surface film, a fluid layer not coagulated by calcium. (3) Plasma-gel layer, underlying the protoplasmic surface film and consisting of a reversibly gelating portion of the granular cytoplasm.

sions from experimental results described in the following pages.

All living cells, so far encountered, normally possess membranous or jelly-like investments, which may be very thick or very thin. These investments are protein-like[3] in animal cells and pectinaceous in plant cells and, generally, are stiffened by calcium and dispersed by sodium or potassium salts. They are extraneous to the underlying protoplasmic body and can be removed without injuring the viability of the protoplasm. In the diagram they are designated as extraneous coats.

Underneath the extraneous coats and distinct from them is a delicate film which invests the protoplasmic body and on which the integrity of the protoplasm depends. In the diagram this film is designated as the protoplasmic surface film.[4]

Underlying the protoplasmic surface film and serving as a mechanical support for it, at least in Echinoderm eggs, is a layer of granular cytoplasm which is stiff in contrast to the fluidity of the more deeply lying cytoplasm in the interior of the egg. In the diagram this layer is designated as the plasma-gel layer. The gel state of this layer in the Echinoderm egg resembles the plasma-gel of the amoeba and is readily reversible. A slight mechanical manipulation of the egg is sufficient to reverse the plasma-gel layer to a fluid state.

With these considerations in mind regarding the constitution of the cellular cortex, a prerequisite for testing the physical state of the protoplasmic surface of Echinoderm eggs is to remove as much as possible of the normally investing, extraneous coatings. This can be done by shak-

[3] Many proteins and some polysaccharides (pectins) have the property of forming cohesive membranes in the presence of calcium.

[4] The term, protoplasmic surface film or simply surface film, suggested several years ago (11), is preferred to plasma membrane since the latter is generally used to designate the physiologically active part which may or may not be a monomolecular layer lying somewhere in or on the surface layer of protoplasm. All that is claimed in this paper for the protoplasmic surface film is to designate a visibly differentiated surface layer which protects the underlying cytoplasm from disintegration.

ing, by washing in Ca- and Mg-free sea-water and by tearing away the coats. These coatings consist mainly of a more or less copious jelly, an underlying vitelline or fertilization membrane and the so-called "hyaline plasma layer" which develops after fertilization as a relatively tough, almost fibrous coat directly on the protoplasmic surface. Various properties were ascribed to the "hyaline plasma layer" by investigators, some of whom have considered it an integral part of the protoplasm. However, the fact that it can be removed entirely without affecting the viability of the egg is sufficient to classify it as an extraneous coat.

The unfertilized egg is relatively easy to denude by washing. The egg may also be torn so as to cause an outflow of the interior which forms a naked exovate. In the case of fertilized eggs the fertilization membrane is removed mechanically and the eggs then placed in Ca- and Mg-free sea-water. The lack of bivalent salts in the medium causes the otherwise coagulable material of the "hyaline plasma layer" to disperse while it is exuding from the egg's surface.

These denuded eggs are viable, they undergo cleavage within the normal time after fertilization and possess the

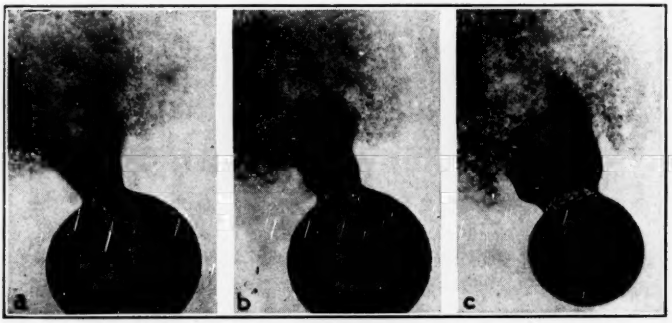


FIG. 3. Denuded, cleaving Arbacia egg in isotonic KCl with one blastomere disintegrating after having been torn with microneedles. Note in three successive views, a, b, c, progressive shrinkage of remaining blastomere with no wrinkling at its surface.

usual properties of permeability, at least in so far as concerns penetrating and non-penetrating dyes.

A marked property of the naked protoplasmic surface is the absence of any sign of wrinkling when the surface is mechanically deformed or is caused to diminish in area. A striking experiment was made by tearing one of two blastomeres of a denuded cleaving egg in a solution of potassium chloride isotonic with sea-water (Fig. 3). While the torn blastomere disintegrated and dispersed in the medium the contents of the other blastomere flowed out through the core of the stalk connecting the two blastomeres. This resulted in a progressive decrease in size of the superficially still intact blastomere, the surface of which maintained a smooth contour as its area diminished.

A denuded egg, made to swell by immersing in hypotonic sea-water, seems to be no more turgid than an egg immersed in normal sea-water. This suggests that the expansion of the protoplasmic surface, caused by the swelling, is not analogous to the stretching of an elastic membrane, but is probably due to the addition of fresh material from within the egg. A state of turgor in cells which has been measured by many investigators presumably depends upon the relatively stiff coatings external to the protoplasm.

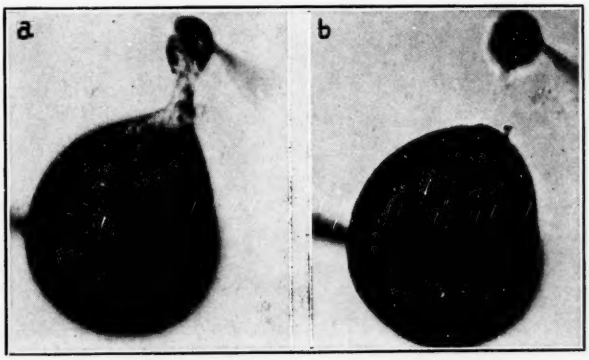
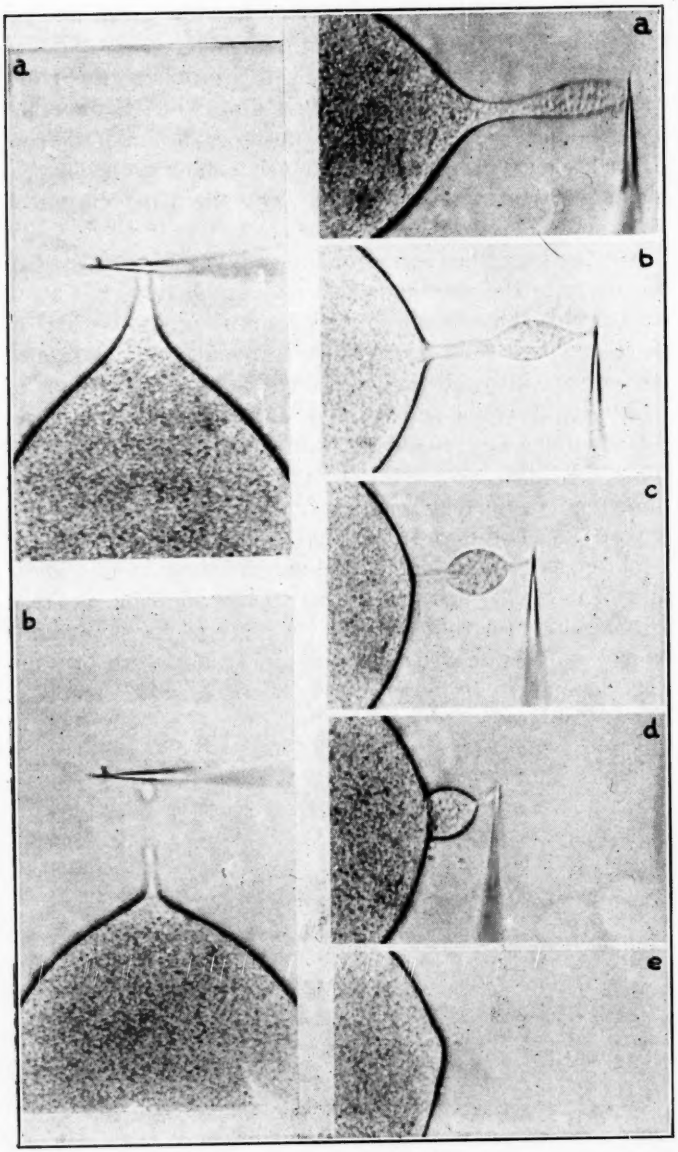


FIG. 4. A mature Arbacia egg denuded in isotonic KCl with drawn-out strand in a, becoming pinched off, in b, without cytolysis.


a
a
b
b
c
d
e

The fluid state of the protoplasmic surface is suggested in experiments of dragging out strands with the tip of a microneedle from the surface of a denuded Arbacia egg or of an artificially produced exovate of a starfish egg. The superficial layer of protoplasm when pulled out with a needle, either pinched off and rounded up immediately (Fig. 4), or was drawn into a lengthening cylinder which broke into spherical beads joined together by a delicate filament (Figs. 5 and 6). As long as this cohering filament persisted, a release of the pull caused the beads to merge with one another and with the protoplasmic body from which the material had originated. No coalescence occurred if the cohering filament broke apart.

Another indication of the fluidity of the protoplasmic surface is the churning movements which can be induced in an unfertilized, mature Arbacia egg denuded of its extraneous coatings. It is significant that this experiment can be done with eggs immersed either in sea-water, in solutions of calcium or of potassium chloride. Eggs previously stratified by centrifugation exhibit the same property. The eggs were mounted in a broad, shallow hanging drop and those adhering to the coverslip pushed gently with a blunt microneedle (Fig. 7). As the egg was pushed along the undersurface of a coverslip, peripheral currents appeared, streaming forward along the two sides and backward along the top and bottom of the egg. Extraneous particles, adhering to the surface, clearly showed by their movement that the streaming involved the surface. A drop of olive oil, applied to the surface which it caps, was carried forward along the side and backward along the undersurface of the egg.

A striking result occurred when the oil-cap was large enough to be so maneuvered, during the pushing of the

FIG. 5. "Beading" of strand drawn out from surface of naked exovate of Asterias egg. Drawn out strand in a, becoming beaded, in b, after several seconds.

FIG. 6. Five successive views of strand, drawn out from surface of starfish exovate, changing shape as strand is allowed, within several seconds, to return and merge with cytoplasm of egg.

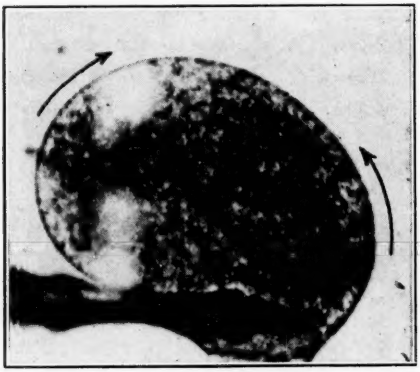


FIG. 7. Denuded unfertilized Arbacia ovum caused to undergo churning movements by pushing egg with side of microneedle.

egg, that it lay over the region including the two opposing, peripheral currents. When the currents once got under way the oil-cap broke in two, each portion being carried along in an opposite direction.

Still another experiment was the following: A drop of oil, exuding from the micropipette but remaining continuous with the oil in the pipette, was brought into contact with the surface which it capped. By moving the pipette the oil slipped over the surface but could not be pulled off without carrying some protoplasm away with it. The cap of oil on the protoplasmic surface behaved like a drop of oil on the surface of water.

Some oils, brought into contact with the protoplasmic surface, fall through it instead of remaining as a cap on the surface (Fig. 8). Recent researches (Chambers and Kopac, 1937; Kopac and Chambers, 1937) have shown that the property of coalescence of an oil-drop with the protoplasm of an Echinoderm egg is a function of the interfacial tension of the oil. The higher the interfacial tension against the sea-water, the greater is the probability of the oil being engulfed by the egg-protoplasm. The important point to be noted here is that the coalescence may occur with no evident disruption of the protoplasmic surface

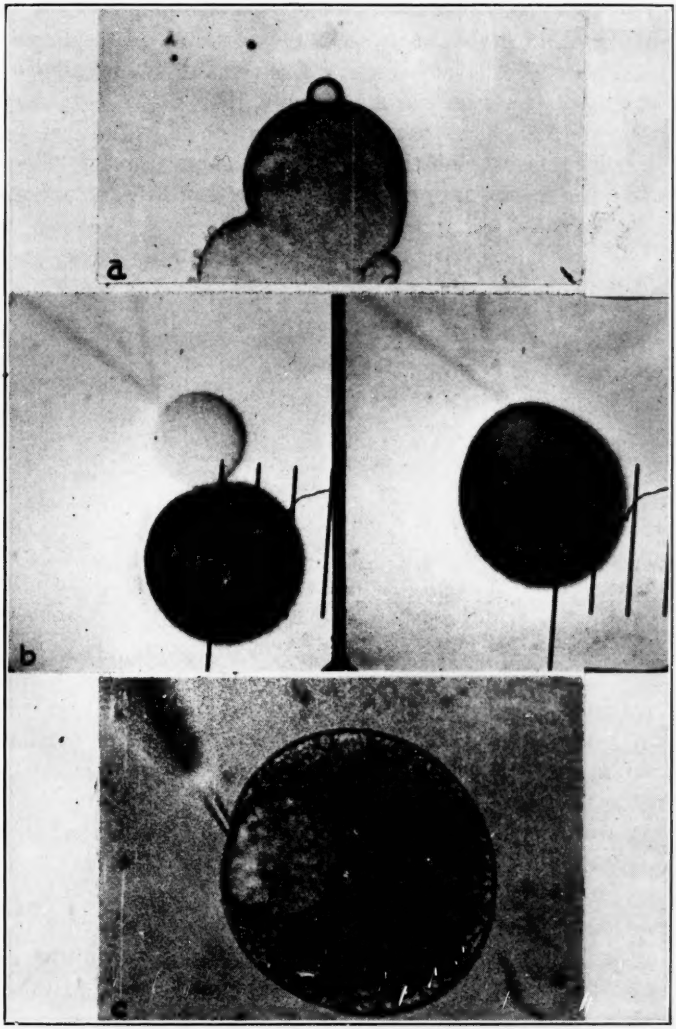


FIG. 8. a. Exovate of mature starfish egg capped with drop of olive oil. b. Two successive views, 1/16 sec. apart, of denuded Arbacia egg showing application and coalescence of paraffin oil drop. The egg shifts its position and swells after coalescence while oil is stationary, being attached to tip of pipette. c. Enlarged view showing coalesced oil drop within egg and flattened against inner surface of protoplasmic surface film.

(*cf.* Fig. 8, b). The oil-drop technique also enables one to distinguish between a coated and a naked protoplasmic surface. An oil-drop, having a given interfacial tension which ensures ready coalescence with a naked egg, may not coalesce with a coated egg or may do so with difficulty, the oil remaining on the surface of the egg for a few seconds and then snapping in by disrupting the extraneous coat.

THE CORTEX OF THE PROTOPLASM

There is a difference between the position of an oil-drop according to whether it has been introduced into an egg by coalescence from without or by direct injection. The coalesced drop lies, for a time, flattened against the inner side of the protoplasmic surface film, while the injected oil is a sphere from the very start, with its boundary always at an appreciable distance from the protoplasmic surface, even when it is subsequently pushed toward the surface from inside the egg. This suggests the existence of a cortical, plasma-gel layer which offers resistance to an injected drop but which either has disappeared or been displaced when the oil enters by coalescence. The presence of such a relatively stiff layer has been indicated also from experiments in which the tip of a microneedle, thrust through the interior of an Arbacia egg to the cortical region at the opposite side and then withdrawn, could be made to drag a strand into the more fluid interior of the egg. Moreover, in Arbacia eggs, centrifuged after being fertilized, a granular cortical layer is plainly visible at the periphery of the hyaline zone. Externally this layer can not be distinguished by ordinary means from the superficial protoplasmic surface film. Internally it grades into the fluid interior of the cytoplasm.

The relatively stiff texture of the plasma-gel layer can be made to reverse to a fluid state without destroying the integrity of the superficially lying protoplasmic film. In the unfertilized Arbacia egg, where the plasma-gel layer is only slightly developed, this can be done by churning

the egg with a microneedle. A localized disappearance of the plasma-gel layer in both unfertilized and fertilized eggs also can be induced by puncturing the egg, pulling out strands of cytoplasm from the surface and releasing them so as to allow the strands to flow back and merge with the surface. Brown (1934) and Marsland (1936) have made some interesting observations indicating that very high hydrostatic pressures induce liquefaction of the cortex.

These various experiments indicate a dissimilarity between the plasma-gel layer and the protoplasmic surface film, the latter constituting the actual external surface of the protoplasm. It has not been possible to lift this surface film by means of needles from the plasma-gel layer. The film is too delicate and fluid to be seized without involving the underlying layer, which tends to liquefy so that cytoplasmic granules are carried along in the strand being pulled out with the needle.

However, nature has provided a means which seems to be the nearest approach to inducing an elevation of the surface film while the underlying layer is still in its normal, jellied state. This occurs during the insemination of the egg. The insemination cone which appears on the egg where the head of a spermatozoon has entered is a minute blister containing a clear liquid invested with a very delicate film-like membrane. These blisters alternately well up and sink with undulating contours and persist for five to six minutes after insemination. In denuded, immature eggs the insemination cones are unusually large and form rounded blisters which, by gentle manipulation with a needle, can be made to spread so as to present the aspect of a delicate film extending over a considerable portion of the egg and separated by a clear liquid from the underlying, granular plasma-gel layer (Fig. 9). This elevated film, when pricked with the tip of a microneedle, breaks down in a wave of disruption from the pricked spot until all the raised film is destroyed. After such an operation cytolysis always occurred, spread-

ing into the interior of the egg from the region of the exposed plasma-gel layer.

These experiments present evidence for the existence of a superficial layer of protoplasm as a water-insoluble, flowing, film-like membrane, which is normally supported, on the inside, by a granular plasma-gel layer, seemingly a more condensed portion of the internal fluid cytoplasm

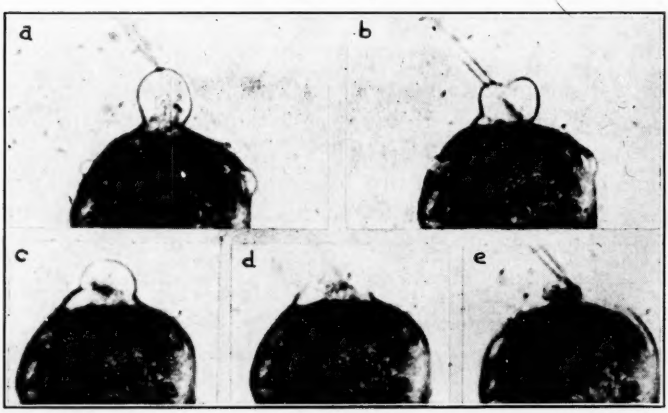


FIG. 9. Enlarged insemination cone or blister on immature Arbacia egg denuded of its extraneous coats. a, b, c. Successive views while blister was being manipulated to induce spreading at base. Note partial escape of granular material from underlying plasma-gel layer into blister. d and e. Momentary views of blister during and after its rupture. The exposed granular cytoplasm is undergoing cytolysis.

and, on the outside, by an extraneous, protein-like cuticle maintained by coagulation when calcium is present in the medium. The cuticle is an extraneous coat because it can be removed without destroying the protoplasm. Likewise, the granular plasma-gel layer, underlying the protoplasmic surface film, can be caused to disappear or to be separated from the film without detriment to the protoplasm. *The structure which alone serves to maintain the integrity of the protoplasm is the delicate, protoplasmic surface film lying between.*

Effect of the Environment on the Plasma Membrane

The protoplasm of the Echinoderm egg remains normal with no evidence of cytolysis as long as the protoplasmic surface film remains intact. In the normal environment of sea-water the protoplasmic surface is very stable and can be extended to a remarkable degree, without losing its continuity. Moreover, if the surface is torn so as to expose the underlying cytoplasm the surface film tends to be rapidly repaired with little loss of cytoplasmic material.

Under the adverse conditions of an environment lacking polyvalent cations (Ca and Mg) the stability of the protoplasmic surface film still is maintained as long as manipulations of the surface do not actually break the film, even though the manipulations may appear to be very severe. For example, the film remains intact and the protoplasm presents a normal appearance if a slow cut be made into the surface, or if the surface be pulled out as strands which may even pinch off and round up, or if the egg is churned so that it seems to be presenting new surface material during the churning.

On the other hand, cytolysis always occurs in a medium lacking calcium if the protoplasmic surface film is broken so that an actual gap is produced by a sudden tear, even though the tear be slight.

It is the repair of an actually broken surface for which calcium in the environment is necessary. At least one of the conditions for the repair seems to be a preliminary, peripheral coagulation of the exposed cytoplasm to constitute a solid layer beneath which the new surface film appears.[5] The inner boundary of the solid coagulum probably serves as an interface where surface-active material may accumulate to form itself into the protecting film.

Calcium enhances the stability and liquid nature of the protoplasmic surface film which at the same time prevents

[5] This peripheral coagulation may be related in some way to the surface precipitation reaction described by Heilbrunn (17).

the calcium in the environment from diffusing into the internal cytoplasm in sufficient amount to induce irreversible coagulation.

Conclusions regarding the cortex of the Echinoderm egg from the experiments described in this paper are as follows:

1. The protoplasmic body of the egg is covered with removable, extraneous coatings.

2. The superficial layer of the protoplasm is a well-differentiated, visible, fluid film distinct and separable from the underlying cytoplasm. Evidences for the fluid nature of this film are: (a) lack of wrinkling when the surface is deformed or diminished in area; (b) pronounced expansibility with no evidence of change in tension; (c) readiness of drawn-out strands to become beaded and to separate into spherical drops; (d) production of surface-currents flowing in opposite directions without rupture of the film; (e) ability of an oil drop, capping the surface, to be slid along without wrinkling the surface; (f) falling through of a coalescing oil drop with no evident rupture of the film.

The protoplasmic body is normal as long as its enclosing surface film is intact, regardless of how much the film is artificially expanded or whether the environment contains or lacks calcium. An actual break of the film results in disintegration which tends to be localized by coagulation of the exposed cytoplasm if calcium is present in the environment, whereupon a new, protecting surface film forms at the interface between the coagulum and the underlying cytoplasm.

3. The plasma-gel layer under the protoplasmic surface film is, experimentally, readily reversible to the sol state.

4. Calcium in the environment stiffens the extraneous coats of the egg but enhances the stability and fluid nature of the protoplasmic surface film and coagulates the plasma-gel layer wherever the film is ruptured.

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A COMPARISON OF THE NERVOUS SYSTEM IN NORMAL AND SINISTRAL SNAILS OF THE SPECIES *CAMPELOMA RUFUM*[1]

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IN normal individuals of the genus Campeloma, the aperture through which the body of the snail emerges is to the right of the axis of the shell. Such individuals are designated as dextral. Van Cleave (1936) has recently studied the reversal of symmetry in *Campeloma rufum* and has found that the numbers of sinistral individuals decrease progressively after the young snails leave the maternal uterus. Symmetry in *Campeloma rufum* is not determined by simple Mendelian inheritance as it is in pulmonate snails. Professor Van Cleave suggested that the writer study the anatomy of normal and sinistral individuals to determine any significant differences that might accompany the gross shifting of parts distinctive of "reversed" individuals. In this study, attention was directed to general organization only incidentally, for it seemed particularly important to determine the effects of reversed symmetry on the nervous system and to study the origin of nerves innervating the various organs of the body.

The writer has been unable to find any references to any similar studies in the literature. Consequently, the present investigation has proceeded from a study of normal dextral individuals to a comparative study of the sinistral type. In the study of normal individuals the works by Baker (1911) on Lymnaea, Annandale and Sewell (1921) on Vivipara, Jones (1933) on *Anguispira alternata,* Bartsch and Quick (1921) on *Zonitoides arboreus,* and the general discussion found in Lankester (1905) have been generally helpful, but due to the specific pattern of the nervous system in *Campeloma rufum* all points of the

[1] Contribution from the Zoological Laboratory of the University of Illinois, No. 515.

gross and finer anatomy had to be checked and repeatedly verified on microscopic dissections.

In primitive Mollusca, the symmetry is strictly bilateral. The group of snails to which the name Streptoneura is applied, and of which Campeloma is an example, represent a highly modified condition. Organs such as gills, the excretory organs and parts of the circulatory system which are paired in the more primitive snails are represented by single organs in the Streptoneura. Furthermore, the arrangement of the parts of the nervous system becomes profoundly altered in these streptoneurous snails.

The streptoneurous type of nervous system is one in which the loop formed by the two visceral commissures is twisted over itself, forming a figure-of-eight. This twisted condition is due to the fact that during the development of the snail there occurs both a longitudinal and vertical torsion. In the embryos of dextral snails, the posterior end of the left side grows faster than the right with the result that the mantle cavity and associated organs undergo a process of torsion to the right through about 180 degrees around a vertical axis and become shifted from a primitively posterior position to one a little beyond the mid-line near the head of the animal. At the same time there is a rotation around a horizontal axis which results in the visceral mass occupying a more dorsal position than before. As a result of this displacement, the long visceral commissures become twisted into a figure-of-eight, the right commissure crossing over the esophagus to the left and becoming the supra-intestinal commissure, and the left one twisting under the esophagus to become the infra-intestinal commissure. Accompanying this forward displacement, there has been a reduction in the paired organs. In dextral snails, the topographically right (morphologically left) half of the circumanal complex disappears, leaving one gill, one auricle and one functional kidney.

While there are some species of snails which are normally sinistral, most of the Streptoneura are typically dextral. Thus in *Campeloma rufum* the majority of in-

dividuals are dextral, while the sinistrals are abnormal and may be regarded as monstrosities. A brief comparison of the general organization of the two types of individuals is given in the following paragraphs.

In the normal (dextral) *Campeloma rufum,* the single gill is located on the left side of the body, though it had its embryological origin on the right side of the embryo and assumed its present position through torsion. In the sinistral specimens of *Campeloma rufum,* the single gill is on the right side of the body and has been developed from a rudiment which would have been completely suppressed if the snail had developed normal dextral symmetry. One of the chief objects of this present investigation has been an attempt to determine differences in the nervous systems of dextral and sinistral individuals of *Campeloma rufum* correlated with the radical change in position of the functional gill and other organs influenced by torsion.

The writer acknowledges her indebtedness to Professor H. J. Van Cleave, who suggested this subject for study and whose generous suggestions and unfailing interest in the work have been of very great assistance. Thanks also are due Miss Lola McClurg for her help in collecting specimens and Dr. N. T. Mattox for his helpful advice.

MATERIAL AND METHODS

The snails used in this study were collected from the Salt Fork River, north of Homer, Illinois, in the same area described by Van Cleave and Altringer (1937: 168).

Several methods of killing were used. Some of the snails were suffocated by placing them for about twenty-four hours in a jar of tepid water from which the air had been driven by boiling. Others were killed by adding magnesium sulfate to the water and leaving the jar stoppered until the snails were dead. The advantage of these methods is that the snails tend to die in an extended condition. Other snails were killed by cracking off the shell without any preliminary treatment. No matter what killing method was used, the snails either were put into Bouin's fixative for twelve to eighteen hours, then into

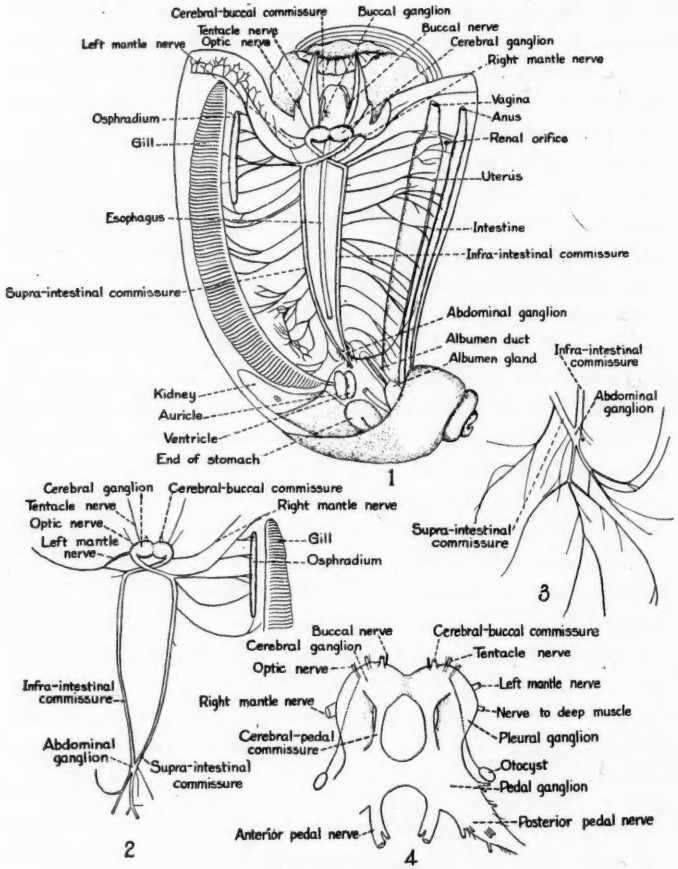
50 per cent. alcohol, and finally 70 per cent. alcohol, in which they were kept, or else were dropped directly into 50 per cent. alcohol and later into 70 per cent. alcohol. Bouin's fixative was preferred because the nerves were left stronger and yellow in color, making them more easily seen. If the preserved snails became too stiff and tough, a little glycerin was added to soften them. The nerves in a fresh snail are almost colorless and are hard to trace, but the internal organs present a far more natural appearance than was observed in specimens which had been preserved. For dissection, the snails, either fresh or preserved, were placed in a parowax dissecting dish made by scooping out a block of parowax until a depression the desired size had been made. This dish has the double advantage of being translucent so that light may come through during the dissection under a binocular microscope, and of permitting the animal to be pinned out with fine insect mounting pins as the dissection proceeds.

A snail pinned firmly through the operculum to the parowax dish is opened by a cut with sharp scissors near the mid-line of the mantle between the gill and intestine. The loosened halves of the mantle are turned one to each side of the animal and pinned down. The antero-dorsal portion of the body is thus exposed as well as the organs on the mantle. The more posterior organs are exposed as dissection proceeds.

Nervous System of Dextral Individuals

Campeloma rufum possesses a well-developed nervous system. The cerebral, pleural and pedal ganglia (Fig. 4) are paired and massed in a ring around the esophagus just back of the pharynx. The two buccal ganglia are located anteriorly on the buccal mass, and the one median abdominal ganglion is posterior to the others and is connected to the pleural ganglia by long visceral commissures (Fig. 1).

The cerebral ganglia are the two most dorsal ganglia of this ring (Figs. 1 and 4). They are located above the esophagus and extend a short distance laterally beyond



EXPLANATION OF PLATE

THE NERVOUS SYSTEM OF CAMPELOMA RUFUM

FIG. 1. Dissection of a dextral individual from the dorsal surface.

FIG. 2. Dissection of a sinistral individual from the dorsal surface. Only the cerebral and abdominal ganglia and some of the main nerves are drawn for comparison with the normal individual shown in Figure 1.

FIG. 3. Detail of nerves from abdominal ganglion of a dextral individual. Dorsal view.

FIG. 4. Anterior view of the nerve ring around esophagus composed of cerebral, pleural and pedal ganglia.

it. These ganglia are large, roughly oval in outline and pink in color. They are joined medially to each other, laterally to the pleural ganglia (Fig. 4), and each is connected by a short commissure to the pedal ganglion beneath and by a longer commissure anteriorly to a buccal ganglion in the head.

Each cerebral ganglion gives off a nerve to the tentacle on that side (Figs. 1 and 4), an optic nerve to the eye and a buccal nerve to the pharynx which arises near the origin of the cerebral-buccal commissure. From the base of each optic nerve a small connection extends laterally and forward to attach to the outer surface of the pedal ganglion. From this attachment the nerve continues to the otocyst, which lies near the lateral aspect of the pedal ganglion. Small nerves arise from the cerebral ganglia to innervate the surrounding muscles, the muscles in the tentacle and the immediate esophageal region.

The pleural ganglia (Fig. 4) lie between the cerebral and pedal ganglia and are not distinctly separated from them. They are brownish in color. From the posterior part of the right pleural ganglion near its junction with the cerebral ganglion arises the supra-intestinal branch of the visceral commissure (Fig. 1). More anteriorly and laterally, a large mantle nerve is given off which joins with a nerve below from the infra-intestinal commissure, and the fused nerves supply the anterior part of the mantle on that side. Smaller nerves, given off the lateral side of this pleural ganglion, supply the muscular tissue in that vicinity. Small nerves from the medial surfaces of these ganglia supply the adjacent region of the esophagus.

The infra-intestinal branch of the visceral commissure (Fig. 1) arises from the posterior aspect of the left pleural ganglion near its junction with the cerebral ganglion above. Near the beginning of this infra-intestinal branch two rather large nerves are given off laterally from the left pleural ganglion which supply the muscles and upper mantle on the left side. The lower nerve lies deep in the muscle tissue, while the more dorsal mantle

nerve runs closer to the surface of the muscle mass and extends on into the mantle. This upper nerve connects with the supra-intestinal nerve below and continues along the upper part of the mantle, where a network of small branches supplies the mantle's edge and a second series of branches identify it posteriorly with the nerve below. Small nerves are given off also from this ganglion to supply the adjacent muscle tissue.

At the point where the supra-intestinal commissure bends posteriorly it gives off a small nerve which joins it to the mantle nerve above and also a large nerve which continues laterally a short distance and then splits into two parts (Fig. 1). The anterior branch passes forward and to the left to supply the anterior region of the osphradium and gill, and continues along the top of the mantle, connecting by numerous branches with the mantle nerve above. The posterior portion of this anterior branchial nerve supplies the middle portion of the osphradium and sends small branches to the gill beyond. Posteriorly, the supra-intestinal commissure gives off a series of branches which innervate the posterior end of the osphradium and then the gill along its length. Near the posterior end of the branchial cavity several nerves combine to form a fine branching network. The heart and pericardial membrane are supplied by nerves from the lower end of this commissure.

The infra-intestinal commissure along its anterior portion early sends off a series of nerves to the right part of the mantle and to the uterus and intestine thereon. The first large nerve (Fig. 1) bends forward and outward to join the mantle nerve above and the conjoined nerves supply the top part of the mantle, becoming continuous with the mantle and anterior branchial nerves from the left side. A series of nerves continue to branch from the early part of this commissure and supply the vagina, external renal orifice and anus, as well as the anterior portions of the uterus and intestine. Along its length, nerves continue to supply the uterus-intestinal region. At its posterior end, several small nerves are given off which

extend around the coil and supply the stomach and other organs in the spire.

The pedal ganglia are located under the esophagus and are brownish in color. They are joined medially with each other, laterally with the pleural ganglia, and are connected with the cerebral ganglia by short thick commissures (Fig. 4). These ganglia innervate the foot of the snail. Dorsally and medially from each arises a large nerve which passes forward to supply the anterior part of the foot. These two large nerves diverge along their course, but a series of branches from their medial surfaces spread forward and inward to form an elaborate anastomosis. The pattern is somewhat asymmetrical. Numerous small branches extend to the anterior margin of the foot. Other distal branches from the two large nerves extend outward to supply the lateral regions in the front part of the foot.

Continuing downward, the pedal ganglia give off branches which supply the near-by muscular tissue and the central part of the foot. Finally, each pedal ganglion continues as a large posterior pedal nerve which by means of its branches serves the posterior region of the foot.

From its attachment on the lateral surface, a small nerve from the cerebral ganglion (Fig. 4) continues on to the otocyst, which lies near and often against the lateral aspect of the pedal ganglion.

Each buccal ganglion is joined to a cerebral ganglion by a rather long flat commissure which extends along the lower aspect of the buccal sac for about two thirds of its length. The commissure then turning upward and passing under some of the muscular tissue emerges again on the dorsal lateral aspect of the buccal mass to join with the buccal ganglion (Fig. 1). Along its forward course, this flat ribbon-like commissure gives off several branches which continue forward and with the buccal nerve innervate the lateral and anterior portions of this region. Branches from the buccal ganglia supply the surrounding tissue.

The abdominal ganglion is located at the lower junction

of the supra-intestinal and infra-intestinal commissures and lies on the right side of the body (Fig. 1). The supra-intestinal commissure crosses over the esophagus in this region. The abdominal ganglion sends nerves to the viscera in the posterior part of the snail and to the anterior part of the mantle in the region of the uterus. Some variation was noted in the branching of the small nerves in this region. The main nerves seem fairly constant.

From the right side of the abdominal ganglion a large nerve continues posteriorly a short distance and then divides into several branches (Fig. 3). The anterior branch curves dorsally and anteriorly and continues on the roof of the mantle cavity under the wall of the uterus. Another branch runs almost parallel to it for a short distance in the region of the intestine. The posterior branch continues ventrally and posteriorly to supply the region of the intestine in that vicinity.

The next large nerve from this ganglion continues posteriorly below the uterus, where some of its branches supply the kidney and others continue beyond to the right. Nerves from the left side of the ganglion run to the kidney and the pericardial region, and though exact details have not been made out they seem to innervate these organs.

Nervous System of Sinistral Individuals

In the sinistral snails (Fig. 2), the arrangement of most of the body organs, the nerve ganglia and the nerve trunks are the exact reverse of their arrangement in the dextral specimens. The ganglia corresponding to the right ganglia in the dextral snails are found on the left side in the sinistral forms and *vice versa*. The left visceral commissure crosses the esophagus to the right and becomes the supra-intestinal commissure, while the commissure from the right pleural ganglion passes under the esophagus and becomes the infra-intestinal commissure. Near its lower end the supra-intestinal commissure crosses back over the esophagus and joins the infra-intestinal commissure and abdominal ganglion on the left side of the body. Main nerve trunks from the various ganglia are exactly

reversed in their position, as would be expected with the reversal in organ location.

So far as dissections have proceeded the arrangement of the smaller nerves and branches in the sinistral individuals follows the exact plan outlined above for the dextral snails but in reverse arrangement of all parts.

Summary and Conclusions

(1) The body of dextral individuals of *Campeloma rufum* has typical streptoneurous arrangement of the nervous system.

(2) The ganglia have the epiathroid type of arrangement, in that the pleural centers are in contact with the cerebral and the pedal ganglia are concentrated anteriorly.

(3) Sinistral individuals show a reversal of organs as well as of shell. The nervous system follows the reversed location of organs. The nervous system has similar arrangement of ganglia and of main nerve trunks as in the dextral individuals, but all structures which occur on the right side of the body of a dextral specimen are transposed to the left in the sinistrals and *vice versa*.

(4) The topographically right gill disappears in normal dextral specimens, while the topographically left one disappears in sinistral ones. Thus the functional gill in the sinistrals is the one that fails to develop in normal dextral individuals.

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TIME OF DETERMINATION AND TIME OF DIFFERENTIATION OF APHID WINGS[1]

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THE times at which the fate of organs is decided play so significant a rôle in development that any information which enables the embryologist or geneticist to set limits within which determination occurs is important. There has been some tendency to regard the time of differentiation as affording a clue to the time of determination, at least to the extent that the order of determination is expected to be the same as the order of differentiation. When the order of visible formation of organs proves to be the same as the theoretical order of determination as ascertained from experiments with intersexes and other intermediates, the embryological evidence is considered to support the conclusions from experiments. And yet, when any part develops slightly out of its calculated turn, it is possible to assume—and this has been done—that a long time elapses between determination and differentiation, and that determination was not after all misplaced. Any opportunity to discover the extent of this time gap should therefore be seized.

Some information has been gained about the *order* of determination of structures in both gamic and parthenogenetic aphids, and the actual time of determination of wings has been set within very narrow limits. Whether a parthenogenetic aphid is to be winged or not is settled about 34 hours before birth (Shull, 1928). This is the middle of the range of a number of observations and may have only statistical significance; for individuals the time may be more than or less than 34 hours before birth. Since the early development of wing buds is histologically

[1] Contribution from the Department of Zoology, University of Michigan. This is one of a series of investigations aided by the American Academy of Arts and Sciences and the Faculty Research Fund of the University of Michigan.

recognizable, it is also possible to observe practically when differentiation starts, thus discovering the lag of differentiation behind determination.

The time of wing determination was first discovered in the strain of *Macrosiphum solanifolii* which came to be referred to in later papers (Shull, 1932) as clone A. The differentiation of wings was to have been timed in that strain; but before this work could be done, clone A underwent the striking "mutation" to clone A′ in the fall of 1929 (Shull, 1932) in which its response to intermittent and constant light was reversed and made much less definite. The study of differentiation was then necessarily postponed until another strain responding in the same, or some other definite, way to an environmental agent could be found. This has been done; the new strain has been subjected to long tests (Shull, 1937) and found to react to light in the same and as sharply defined manner as did the older clone A. It is in this clone that the study of wing rudiments has been made.

Source of Winged and Wingless Individuals

Since determination of wings occurs before birth, it would be necessary to examine unborn embryos if differentiation of wings promptly follows their determination, or early instars if differentiation is moderately delayed. Externally, wings are not recognizable by ordinary means until the third instar, so some other way of knowing which individuals will be winged must be available. Moreover, it must be possible, if the comparison is not to resolve itself into a vicious circle, to say that a certain young individual would become winged as an adult no matter what the histological or other examination showed.

Fortunately, the experimental results indicate a sharp criterion. At the same time that young aphids were reared for sectioning, others were raised in the same way and kept to maturity. From these controls it could be assumed with considerable confidence that the offspring of a wingless mother kept in alternating light and dark-

ness at 14°, if born four to six days after the treatment began, were destined to be winged. In like manner it was practically assured that the offspring of a winged mother reared at 24° in continuous light would be wingless. Aphids treated in other ways have been studied, but all mentioned in this paper have come from one of the two sources described.

THE WING RUDIMENTS

The wings start as thickenings of the hypodermis in the dorsolateral regions of the thorax. There are two on each side, anterior and posterior. The former, when first certainly recognizable, forms a patch about 65 μ in diameter whose center is about 110 μ behind the posterior margin of the compound eye. The posterior rudiment is recognized as a patch about 25 μ in diameter, with its center approximately 150 μ behind the compound eye. The hypodermis becomes gradually thicker from the edge of the rudiment inward; this increase sometimes continues to the center, producing a distinct peak, sometimes the central part is more nearly flat. The hypodermis thins out between the anterior and posterior wing buds, but is seldom as thin there as elsewhere in the wall of the thorax. Thus the two buds of the same side almost merge into one, but there is a depression between them. In the thickening of the hypodermis in these areas there is soon apparently also tangential growth, for the layer of cells becomes bent. The bend is frequently double, and could be regarded as one invagination and one evagination. Eventually, of course, the protrusion is outward.

When the wing rudiments are studied in serial transverse sections, the gradual increase in thickness and its gradual recession in successive sections, then a briefer repetition of the thickened series behind it, are marks which help to distinguish the buds from other slight thickenings. Their position relative to the eyes is likewise a recognition mark.

Nature of Wing Buds at Various Postnatal Stages

When wings are detectable externally, in the third instar, they are already deep pouches projecting downward and backward from their attachment to the thorax. In the second instar they are likewise distinct sacs. In the first instar they are unmistakable, though seldom is there much evagination of them. The thickened layer may appear sharply bent in one or two places in a section, producing the doubtful inward or outward protrusions described in the preceding section. Every first instar examined, which had been so treated as to lead to the expectation that it would be winged, had these patches of thickened hypodermis. Even those which were fixed less than an hour after birth had them distinctly, though frequently the layer was not sharply bent at this stage.

It is clear from these observations that the beginnings of the wing rudiments must be sought in the unborn aphids.

The Age of Embryos

To trace wing buds back into the prenatal stages, it becomes necessary to determine the relative ages of the embryos. The early stages can easily be recognized by the cleavage, germ band, revolution of the embryo and formation of the appendages and eyes. When most of the structures have been laid down, the principal recognition mark is size, and this is an uncertain standard. As the embryo approaches term, it becomes less compact and stains less heavily. In a general way the older embryos are in the posterior part of the abdomen, the younger ones forward; but with the branching form of the reproductive system and the crowding of the embryos and organs of the mother, position is of little use as a guide. In one individual, a germarium, which ought theoretically to be in an anterior position, was actually almost at the base of the cauda. Among the older (less compact, more lightly stained) embryos, eye pigment furnishes an additional criterion. Of the embryos which, on the basis of size,

looseness and light staining, might be regarded as of about the same age, some have pigment in the compound eye, others none. Pigment begins to form in the central ommatidia, spreads to other ommatidia and becomes more dense. All aphids have pigmented eyes at birth. Not all the ommatidia are distinctly red then, and whether aphids vary much in this respect at birth has not been ascertained. But even if there be considerable variation in the degree of eye pigmentation, it seems likely that the pigment is as good an indication of age (stage of development) as birth is. Half a dozen embryos may lie at the base of their respective ovarioles, in a position where they might move on down the oviduct and vagina. What causes one individual to be born in advance of the others may conceivably be something wholly outside its own development or physiology. Abnormal conditions may prevent aphids from being born, and the body of the mother becomes swollen with her accumulating offspring. One can scarcely assume that the embryos remain "young" because of such stoppage of birth. Eye pigment, therefore, is probably a satisfactory criterion of age in the very late prenatal stages.

First Appearance of Wing Buds

In embryos of intermediate age, but after most of the main structures are laid down, the hypodermis is relatively thick, thicker than in later stages. This thickness is subject to many irregularities. Thicker places are scattered here and there over both thorax and abdomen. But none of these early thickenings is of any extent. They do not occur with increasing distinctness and then fade out in a series of successive sections. Most of them are not located where wing rudiments develop. It is plain that wings have not commenced their differentiation at this intermediate stage.

Even among the larger, looser, less stained embryos, those without any red pigment in the compound eyes have never been found to have any thicker hypodermis in the wing bud region than do embryos destined to be wingless.

When the first trace of red pigment is found in the eyes there is still no additional thickening in the wing area, as judged from embryos destined to be wingless as a standard. But with approximately one fourth of the ommatidia pigmented, no specimen yet studied has failed to show the characteristic wing development if its treatment has been such as to call forth wings (intermittent light, 14° temperature). The thickening of the hypodermis in such embryos has its front edge about 75 μ behind the eyes, gradually increases in degree in successive sections and then diminishes back of that point, to form a patch some 65 μ in diameter. At this stage of one-fourth pigmented eyes, however, there is usually no posterior wing rudiment. One such embryo had a suggestion of a posterior thickening which might not really have been a wing bud.

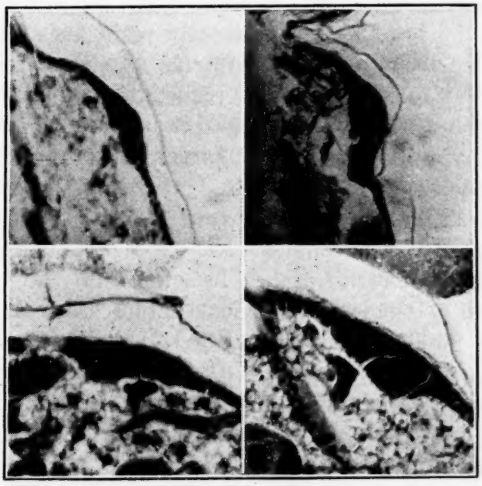

FIG. 1. Wing buds of young aphids. *Upper left*, first instar wingless aphid less than one hour after birth; *upper right*, first instar winged aphid less than one hour after birth; *lower left*, unborn winged aphid with eyes scarcely half pigmented; *lower right*, unborn winged aphid with eyes distinctly more than half pigmented. The upper two are equally magnified, as are also the lower two; but the enlargement of the lower two is greater than that of the upper two.

In prospective winged embryos with fully half the ommatidia pigmented there is always a plain anterior rudiment (Fig. 1, lower right) and usually a posterior one. Greater degrees of eye pigmentation are always accompanied by the regular complement of four wing buds.

It must be concluded from these observations that wing differentiation starts very soon after pigmentation of the eyes commences.

WING RUDIMENTS IN WINGLESS APHIDS

In judging of wing development it has been necessary to use presumptive wingless aphids as a standard of comparison. An embryo with its hypodermis thicker in the wing region than elsewhere in the thorax is not necessarily destined to be winged. For embryos which, from the light and temperature treatment they have received, should be wingless nevertheless have such thickenings (Fig. 1). That they are correctly regarded as wingless is abundantly indicated by controls which are treated in the same way and are allowed to reach maturity, and which practically without exception are then wingless. But the hypodermal thickenings are not as thick as those of prospective winged aphids. The wing rudiments of wingless aphids are moreover not bent as a result of lateral expansion in the marked way that genuine wing rudiments are. The difference between the wing buds of embryonic winged and wingless aphids is great enough that one could usually say, after taking into account the stage of development as ascertained from the degree of eye pigmentation, which kind is under observation without looking up the treatment which had been given to it.

Even after birth these thickenings may still be, but are not always, present in wingless aphids. Some first instar wingless aphids reveal no thickenings at all in the wing region, but others have them. When present they show the same crescendo and diminuendo through successive sections. But the hypodermis is much less thick, and is never sharply bent as if for evagination. Whatever

curvature the thickened hypodermis possesses appears to be merely due to the form of the thorax or to shrinkage in fixation. Not infrequently the posterior wing rudiment is wanting when the anterior one is unmistakable. There is on the whole distinctly less uncertainty in the distinction between winged and wingless in the first instar than in late prenatal stages. In later instars the thickening in wingless aphids disappears completely. Indeed, in the second instar none has yet been found, though the number of individuals examined has been small; and in the third instar, which has been abundantly studied, it certainly is lacking.

Time of Development of Eye Pigment

The crucial point in all these observations is the time at which wing differentiation begins. Since this development very closely follows the first production of eye pigment and since the pigment can be observed with much less labor than that required for hypodermal thickening, it is proposed to determine the time of beginning eye pigmentation. The desired time of wing differentiation will be only very slightly later.

From an examination of a number of adult wingless females which were reared in intermittent light at 14° it was found that the mean number of contained embryos with red eye pigment was 2.50. How rapidly these embryos proceed to birth is shown by the breeding records. The exact time, to the nearest five minutes, at which the parents were put on a plant and when they were removed, the number of parents put on and the number found alive at removal, and the number of offspring born to them in the interval were all recorded. From these records it appeared that, to wingless mothers in intermittent light at 14°, an average of 2.31 offspring per female per day were born. The time elapsing between the first formation of eye pigment and birth would thus be $\frac{2.50}{2.31}$ or about 1.08 days. Since wing rudiments begin to thicken shortly

after eye pigment begins to form, it may be estimated that perhaps wing differentiation starts 22 to 24 hours before birth.

A similar calculation for winged parents reared in continuous light at 24° showed that eye pigment starts to form 1.46 days before birth; but whether wing differentiation as quickly follows eye pigmentation under these conditions can not be ascertained from the materials used for study, since no winged offspring are to be expected from such parents. It is conceivable that the eye pigmentation is speeded up more than wing differentiation at the high temperature and in continuous light. Experiments at other temperatures will be needed to test this possibility.

LAG OF DIFFERENTIATION BEHIND DETERMINATION

Adopting 34 hours before birth as the time of determination of wings, and accepting 22 to 24 hours before birth as the time of their initial differentiation, one would conclude that differentiation lags 10 or 12 hours behind determination for these structures. Perhaps this statement is needlessly precise; there is enough fluctuation of the observations on which it is based to permit deviation of a few hours on either side of the time stated. In any event, however, it must be recognized that differentiation is rather prompt, once it has been decided upon.

WINGS IN EVOLUTION OF APHIDS

The existence of wing thickenings in the late prenatal stages and first instar of wingless aphids suggests that the natural tendency of aphids is to have wings, and that development of these structures is stopped by continuous light and high temperature. It points to a primitive winged condition—primitive, that is, with respect to the aphid group. This conclusion is in agreement with what is probably the general opinion of entomologists and others concerning the evolution of wings among the plant lice.

Summary

The time of determination of wings in aphids has been known. Since the early stages of wing development consist of thickenings of the hypodermis in four definite areas, the time of differentiation can be rather accurately decided. Whether an aphid is to be winged or wingless can be known almost with certainty from the treatment (light and temperature) it has received. Embryos shortly before birth, and the first instar, have the wing rudiments in characteristic position even if they are destined to be wingless; but they are thinner and grow less laterally than do those of winged individuals. The additional thickening and the tangential growth which marks future wings starts shortly before birth. The first signs of such development are found in embryos which have already started to form red pigment in the eyes. From the number of red-eyed embryos in a female and the birth rate of her offspring, it is concluded that pigment formation starts about 26 hours before birth, on the average. Wing buds therefore start their definitive thickening perhaps 22 to 24 hours before birth. This is only a few hours after the determination of wings, as ascertained from earlier experiments.

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SHORTER ARTICLES AND DISCUSSION

THE SEX RATIO IN THE CROSS OF PHASIANUS TORQUATUS ♀ × GALLUS DOMESTICUS ♂

It has been reported for various species and genus crosses of birds that among the F_1-hybrids males predominate numerically or that only males occur. As far as can be judged from the evidence available in the literature, to be discussed subsequently, such a situation exists among hybrids of chickens and pheasants. With a single exception (Smith, 1912), all recorded hybrids between these two genera either are known to have come from the cross of chicken ♀ × pheasant ♂, or may be safely assumed to have resulted from this type of mating. It seemed of interest, therefore, to secure data relative to the sex ratio in the reciprocal cross, *i.e.*, pheasant ♀ × chicken ♂.

Since natural hybridization occurs only rarely between the two genera, crosses of this kind became only practical with the elaboration of a satisfactory technique of artificial insemination (Burrows and Quinn, 1935; Quinn and Burrows, 1936). Taking advantage of this new tool, we have made crosses between ringneck pheasant ♀♀ and chicken ♂♂. In 1936 we used Creeper roosters as male parents and White Leghorn roosters in 1937. The sex was determined of all unhatched hybrid embryos which showed sufficient development, and of all birds which died after hatching or which were killed at later stages. The data thus obtained are shown in Table 1. It is evident that among the offspring of this cross the sex ratio is normal, *i.e.*, very close to equality. Among twenty-two hybrids obtained and sexed in 1936

TABLE 1

SEX RATIO DATA FROM PHASIANUS TORQUATUS ♀ × GALLUS DOMESTICUS ♂ HYBRIDS

	Pheasant ♀ × Creeper ♂		Pheasant ♀ × Leghorn ♂		Totals	
	Males	Females	Males	Females	Males	Females
Dead embryos	3	7	22	26	25	33
Dead chicks	3	5	17	19	20	24
Dead adult birds	..	..	1	3	1	3
Total dead	**6**	**12**	**40**	**48**	**46**	**60**
Chicks killed at 0–3 days	..	..	15	8	15	8
Chicks killed later	4	..	1	1	5	1
Total killed	**4**	**0**	**16**	**9**	**20**	**9**
Total sexed	**10**	**12**	**56**	**57**	**66**	**69**

there were ten males and twelve females; among 113 hybrids from the 1937 experiment fifty-six were males and fifty-seven females. The whole material consisted of 135 hybrids, sixty-six being males and sixty-nine females. All hybrids which were hatched in 1936 are included in the table; from the 1937 hatches, on the other hand, twenty animals are still living and unsexed at the time of writing. There is no reason to believe that the remaining animals show a sex distribution different from those already sexed. The deviation from equality within the sub-groups of hybrids which died and of those which were killed are statistically not significant.

As we have indicated already, the situation is very different in the reciprocal cross. The sex distribution of hybrids from matings of chicken ♀♀ × pheasant ♂♂ has been found as shown in Table 2.

TABLE 2

Author	Total number of hybrids	♀♀	♂♂
Cutler (1918)	12	0	12
Poll (1921)	16	6	10
Sandnes and Landauer ..	1	0	1
Total	**29**	**6**	**23**

In a total of twenty-nine hybrids from this cross there were 79.3 per cent. males. This amounts to a deviation of 29.3 ± 9.3 per cent. from equality. Moreover, it is most likely that the hybrids recorded by Guyer (1909) and by Taibell (1932) also came from this type of mating. Guyer's material consisted of twelve hybrids (plus one of unknown sex), and Taibell reported on two hybrids. All were males. If these are added to the previous total, we have forty-three hybrids with a sex distribution of thirty-seven males and six females. There are 86.0 per cent. males in this total, a deviation of 36.0 ± 7.6 per cent. from equality. Thus, there is an undoubted excess of males among the offspring of crosses between chicken ♀♀ and pheasant ♂♂.

An increased percentage of males has been found in the F_1-generation of other genus or species crosses of birds. Haig Thomas and Huxley (1927) reported a preponderance of males in various species crosses of pheasants; Riddle (1925, 1936) and Cole and Painter (1932) made similar observations in pigeon crosses; Guyer (1909) and Ghigi (1936) found that only male hybrids appear in crosses between the domestic fowl and the guinea fowl; the same appears to be true for crosses of guinea fowl and pea-fowl (Ghigi, 1936); and there are some further, less well-authenticated cases of

a similar nature. It appears, however, that only in one instance are data available which can be used for a comparison of the sex ratio among first generation hybrids from *reciprocal* crosses. Among thirty-five sexed hybrids of the cross *Thaumalea amhersti* ♀ × *T. picta* ♂ Haig Thomas and Huxley found twenty-three males, *i.e.*, 65.7 per cent., while in the reciprocal cross they had twenty-nine males (58.0 per cent.) among fifty sexed hybrids. The authors suggest that this result agrees with what may be expected on the basis of Goldschmidt's hypothesis concerning strong and weak sex-determining factors.

Among insects a considerable number of cases is known in which differences in the sex ratio of the offspring occur in reciprocal genus or species crosses. We refer to the observations of Harrison (1919, 1920) on crosses of *Nyssia zonaria* × *Lycia hirtaria, Nyssia zonaria* × *Poecilopsis pomonaria, Nyssia zonaria* × *Poecilopsis lapponaria,* and *Tephrosia bistortata* × *Tephrosia crepuscularia;* of Federley (1929) on crosses of *Chaerocampa elpenor* × *Metopsilus porcellus;* of Bytinski-Salz and Günther (1930) on crosses of *Celerio galii* × *Celerio euphorbiae;* of Sturtevant (1920) on crosses of *Drosophila simulans* × *D. melanogaster;* and of Dobzhansky (1935) on crosses of *Drosophila miranda* × *D. pseudoobscura.* Goldschmidt's results with crosses of different strains of *Lymantria dispar* also belong here. To these cases of differences in the sex ratio among the progenies of reciprocal crosses we may add, as the first clearcut instance among birds, the reciprocal crosses between pheasants and chickens.

Various interpretations have been offered to explain why these reciprocal genus or species crosses give different sex ratios among their progeny. We do not need to discuss these interpretations in detail, since our evidence does not contribute anything towards a causal explanation. The majority of cases at present can be explained most satisfactorily by the assumption of a lack of balance between the X and Y chromosome in one of the two possible combinations (Federley; Bytinski-Salz and Günther). Whatever may be the first cause for the disturbance of the sex ratio in such crosses, the result may be brought about either by differential mortality or by sex reversal. We do not know at present which one of these two mechanisms leads to the prevalence of males among the hybrids of chicken ♀♀ × pheasant ♂♂. It is important, however, to point out in this connection that in the reciprocal cross, i.e., pheasant ♀ × chicken ♂, a high embryonic mortality (43.0 per cent. of 328 fertile eggs) occurred during the

first week of incubation without resulting in an abnormal sex ratio.

Further observations on our material will be published elsewhere.

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ON THE USE OF *DROSOPHILA MELANOGASTER* AS A TOOL IN THE STUDY OF DELAYED KILLING BY X-RAYS[1]

COLLINS and Maxwell (1936) have recently reported observations on delayed killing of seedlings by x-rays. These authors subjected dry seeds of maize and related species to x-ray dosages ranging from 1,000 to 2,400,000 röntgens. Doses up to 60,000 *r* did not affect germination but killed an increasing percentage of plants in the seedling stage. Above 60,000 *r* germination was inhibited the more, the higher the x-ray dose. The dose which inhibited germination completely was estimated as 2,000,000 *r* units.

The experiments of Collins and Maxwell were repeated in our laboratories on a variety of garden seeds with x-ray doses from 2,000 to 120,000 *r* units, and we are able to confirm the delayed killing effect reported by these workers. We also noted that the delayed killing effect is generally more pronounced in quickly germinating seeds. Our experiments were designed to test the influence of temperature on x-ray action. Detailed results will be published elsewhere.

For a closer study of the phenomenon of delayed killing we have chosen the fruit-fly *Drosophila melanogaster* because we are more familiar with the behavior of this material after x-ray treatment and because *Drosophila* seems to offer better opportunities for a detailed explanation of the phenomenon than do seedlings. It is entirely possible, however, that in *Drosophila* the delayed killing may not be due to the same processes which cause a similar type of occurrence in seedlings.

We have defined delayed killing in *Drosophila* as the failure of x-rayed larvae to complete development and emerge from the pupa case. A certain percentage of larvae will die shortly after exposure to x-rays; these are not included in the category of delayed killing, but only larvae which live apparently normally after treatment, pupate normally, but fail to emerge from the pupa case.

In *Drosophila* the lethal x-ray dose is much smaller than with seeds, which constitutes an added advantage of this material in experimental work. Thus in Table I are given the number of eggs and the corresponding number of imagines produced in an

[1] From the Biological Laboratories, Harvard University, and the Haskins Laboratory, Union College, Schenectady, New York.

TABLE I*

Dose	Eosin			Bar			Wild		
240 r				480	449	93.5	380	367	96.5
720 r	1,200	978	81.5	1,000	964	94.6	550	460	83.5
960 r							100	68	68.0
1,200 r	425	95	22.2	1,000	316	31.6			
1,440 r	350	31	8.9	200	14	7.0	210	19	9.0
1,680 r							240	15	6.2
1,920 r	625	3	0.5				200	7	3.5
2,160 r							400	5	1.2

	Notch[8]			White			Brown		
240 r	500	358	71.6						
720 r	720	130	18.0						
960 r	120	13	11.0						
1,200 r	150	0	0.0	100	26	26.0	80	13	16.0
1,440 r	240	1	0.4	160	10	6.0	80	6	7.0
1,680 r									
1,920 r									
2,160 r									

* Various strains of Drosophila were exposed to x-rays as young larvae. The number of eggs was estimated from the egg density, the emerging flies were counted under ether. For each race the first column shows the number of eggs, the second column the number of flies emerged from these eggs, the third column the *per cent.* survivals.

experiment where young larvae, 18 to 22 hours after egg deposition, were exposed to graded x-ray doses. The table represents the results of an exploratory study of the radio-sensitivity of various races of *Drosophila* and does not give information relating to the percentage of flies which reached the pupal stage but failed to complete metamorphosis. It indicates, however, that for several genetically different strains the lethal dose varies from 1,440 to 1,920 *r* units, except for the Notch 8 stock which succumbs at a markedly lower dose. Details of the experiment are available in an earlier communication (Haskins and Enzmann, in press).

For a closer study of delayed killing in *Drosophila* three types of information are available from the present experiment, including: (1) Statistical data on the number of eggs deposited, the number of larvae alive at a specified time after treatment, the number of pupae formed by x-rayed larvae and finally the number of emerging flies; (2) information derived from the behavior of imagines preparing to leave the pupa case, behavior of living flies developing from x-rayed larvae and microscopic examination of the same flies after death; (3) information derived from examination of the contents of pupa cases which failed to produce flies.

Statistical data concerning the percentage of delayed killing are shown in Table II. From this table some information may be

gained relating to "immediate" killing. It will be noted that contrary to the findings on x-rayed seeds, some larvae are killed with all x-ray doses used. The percentage of larvae killed depends

TABLE II*

Dose in r units	Eggs	Larvae before raying	Per cent.	Larvae 24 hours after raying	Per cent.	Pupae (Estim.)	Per cent.	Flies	Per cent.
240	81	79	100.0	79	100.0	71	87.7	68	84.0
720	166	163	95.5	161	91.0	115	90.5	81	48.8
1,200	85	85	100.0	79	92.7	64	75.5	23	27.0
1,680	127	126	99.5	114	90.0	42	33.0	6	4.7
1,920	202	199	97.5	183	89.7	62	30.2	2	1.0

* Larvae of a wild strain of *Drosophila* were exposed to x-rays of various doses at the age of 18 to 22 hours after egg deposition. Counts were made of the number of eggs, the number of living larvae before x-raying, of living larvae 24 hours after x-raying, of pupal and of emerging flies. The larvae were counted by removing them one by one to a new culture. The pupae formed on the sides of the culture bottles could be counted, but those on top of the culture medium could only be estimated. All survival percentages are figured on the basis of the number of eggs being 100 *per cent.*

on the dosage of irradiation given. There is no clear-cut dividing line between those doses which only prevent emergence and doses which kill larvae. Even the smallest x-ray dose used produces both effects. In every case, small x-ray doses cause a greater number of fatalities at emergence than during larval life. A typical experiment is described below.

A culture was supplied with a small number (28) of x-rayed larvae in order to reduce the variability required for various steps during metamorphosis. A control culture reared under identical conditions but not x-rayed supplied a check on the time required for development. It is usually possible, by mere inspection of a culture bottle to determine whether the contents of pupa cases are dead. Dead pupae differ in appearance from living ones and become easily infected with fungi while living pupa resist such infection.

The twenty-eight experimentally x-rayed larvae formed twelve pupae which were opened with dissection needles after the normal time required for metamorphosis had elapsed. All the pupa cases contained living flies, four of which were badly malformed. The failure of the rest to emerge must have been due to other than microscopically visible physiological causes. Contributory evidence may be obtained by an examination of the behavior of living flies from x-rayed larvae, as well as from microscopic studies of etherized material. When flies of the more resistant strains, which had been exposed to larger but sublethal doses of x-rays as

young larvae, were studied, practically 100 per cent. of imagines were found to be abnormal. Some exhibited structural defects in wings, legs, abdomen, bristles, etc., of the nature described by us in earlier papers; a greater number showed no visible structural changes, but it was noticed that the wings were held in peculiarly fixed abnormal positions and that the animals evinced difficulty in flying or walking. The abnormal position of the wings especially suggests that groups of muscles or nerves are affected.

Incidental detailed observations made during a study of the process of emergence in order to determine the onset of the respiratory movements in newly hatched flies give interesting evidence of the cause in special cases for failure of hatching and delayed killing. Thus in one instance a fly was observed which had succeeded in extracting itself partly from the pupa case and had started respiratory motions before it was completely free. The liquid adherent to the pupa case was sucked into the tracheae and deposited inside the wings, causing them to bulge like balloons. The liquid disappeared in time, but the wings shriveled to abnormal shapes. Again, a fly developed from an x-rayed larva was observed during hatching. The insect possessed a large melanotic tumor in place of a right wing. This fly succeeded in freeing the head and thorax but died before it could complete emergence. Various cases of "hatching cripples" were observed. In these insects x-ray-induced disturbances of development have caused the head, thorax and abdomen to be out of alignment, the abdomen being joined to the thorax at a blunt angle, a malformation which seems to be a serious obstacle to emergence. Long delay in emergence is of very frequent occurrence. In several other cases observed the emerging flies showed no external modifications but behaved as if their muscles were too weak to free the abdomen from the pupa case, an act which is performed by normal flies in a few recurrent spasms of peristaltic movement during which the abdomen becomes long and thin and finally slides easily from its case. These observations and many others substantiate the belief that delayed killing in *Drosophila* is essentially an indirect effect of initial x-ray malformations which result in imaginal deformities which hinder emergence. *Drosophila* larvae are in a sense dual individuals. The larva is a complete organism possessed of peculiar larval organs. The imago, however, is represented within the larva in the form of discrete elements, the "Keimscheiben." The imaginal elements develop

rapidly during pupation at the expense of broken-down larval tissue as well as of material stored by the larva. The imaginal rudiments finally coalesce and form the imago. Some larval organs, however, are taken over into the imago without much change.

At the time when the larva is exposed to x-ray, in our experiments, the larval cells have nearly reached a stationary stage in the sense that most of them increase in cell size but not in cell number in the course of further larval growth. The anterior spiracles are an exception to this general condition, and it is noteworthy that they behave after x-ray treatment much more like imaginal elements (Enzmann and Haskins, in press). Imaginal elements in contrast to larval ones grow by proliferation (of nuclei or cells) rather than by increase in cell size.

This difference in the growth tendency between larval and imaginal elements seems to be chiefly responsible for their remarkable difference in sensitivity to x-rays. Larval cells are relatively insensitive, as evidenced by the small percentage of larvae succumbing shortly after radiation treatment. Imaginal cells are very sensitive to x-rays. The chief difference between the two types of elements lies in their mitotic activity. It is possible that the chromosomal apparatus of larval cells may be affected by x-rays as much as that of imaginal nuclei, but the larval chromosomal apparatus is not called upon to function in either chromosome duplication or segregation for mitosis, and the radiation damage inflicted upon it does not therefore lead to serious consequences. In the imaginal nuclei (or cells) of the young larva x-ray induced damage leads to abnormal mitoses and may result in death of daughter cells and deletion of whole organs from the imago, if the x-ray treatment was applied at an early stage of development of the organ in question.

Collins and Maxwell, in discussing the delayed killing of seedlings, consider the derangement of the mitotic apparatus to be the probable cause. The same mechanism is probably the major cause for delayed killing in *Drosophila*. There is in the extensive literature on x-ray effects nothing which requires a different view. Point mutations leading to abnormal development in vital organs and causing delayed death are a possibility, but are not frequent enough to explain our results. Lethal mutations induced by x-ray treatment may conveniently be grouped with chromosomal derangements.

The great experimental advantage of *Drosophila* larvae as the objects of a study of delayed x-ray killing seems to be in the fact that here we are dealing with two clearly separated kinds of cells, one which is vital for the individual at the moment and is relatively insensitive to x-rays, and another which is highly sensitive, but not of great importance at the instant for the continuation of the life of the organism, but becomes so at some point later in development.

The fates of both these kinds of cells may be observed over a very considerable portion of the life of the larva, and by the time it has passed the prepupal stage, the distinction between x-ray effects upon larval (essentially embryonic) and adult tissue may be very clearly drawn, and their interdependence and relative importance in bringing about imaginal death very completely determined.

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OPPOSITE EFFECT OF ENVIRONMENTAL FACTORS ON SIMILAR PHENOTYPES[1]

A classical example of the variable expression of a gene under the influence of a certain environmental factor was described by Morgan (1915). The dominant, sex-linked gene, "Abnormal Abdomen," in *Drosophila melanogaster*, which causes an incomplete chitinization of the abdomen, was shown to manifest itself differently according to different constitution of the food. In a very variable expression the abnormal phenotype usually appears only in moist food, and only those flies hatching early show a good manifestation.

I have worked with a gene a-III found during inbred-experiments in a wild population by Timoféeff-Ressovsky, which produces a phenotype very much like that of the old "Abnormal Abdomen." However, this new gene is recessive and autosomal (according to K. Schaeffer in Chromosome III, near to sepia). Flies homozygous for a-III possess abdomina which vary from fully normal chitinization to extreme lack of it. Four classes were distinguished, as represented in Fig. 1. To study the variation of the manifestation of this gene under different food-condi-

[1] Work done at the Kaiser-Wilhelm-Institut f. Biologie, Dept. R. Goldschmidt. Acknowledgments for help and advice are due to Professor Dr. R. Goldschmidt, Drs. C. Stern and N. W. Timoféeff-Ressovsky.

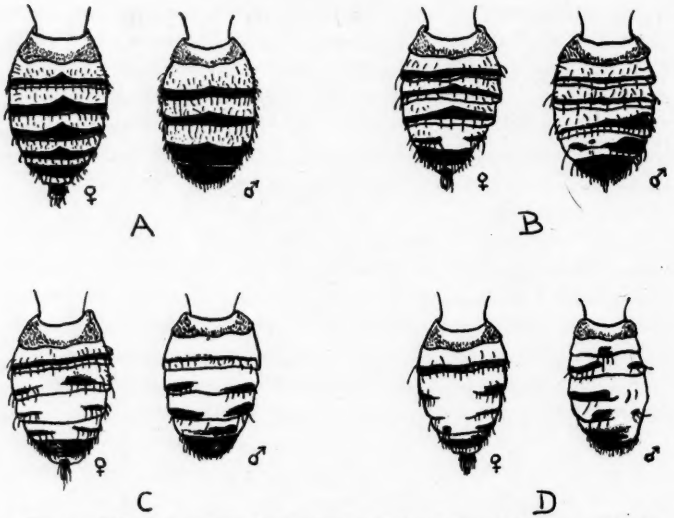

FIG. 1. Different degrees of manifestation of the gene a-III. A = no manifestation, normal. B = poor manifestation. C = good manifestation. D = Excellent manifestation.

tions a proposition of Timoféeff-Ressovsky was followed, calling the percentage of individuals exhibiting any degree of abnormality the penetrance of the gene, while the percentage of flies belonging to class C and D among all flies showing any manifestation was called expressivity. With the help of these two subdivisions it is easy to show the degree of manifestation under different environmental conditions, as which different food-conditions were chosen. The standard agar-agar-cornmeal-molasses mixture served as normal food. The degree of moistness was changed by addition or withdrawal of water during the cooking of the food.

The result of single pair-matings (1 ♀ a-III× 2 ♂♂ a-III) under different food-conditions is shown in Table I.

We see that the phenotypical manifestation of the gene a-III varies greatly according to the food-conditions and that its penetrance increases under the following food-conditions: dry food, old food and small amount of food. A statistically proved high correlation between an increase in the size of flies and degree of manifestation demonstrated that lack of food provides the necessary condition for the penetrance of a-III. The observation that

TABLE I

Condition of food	Total amount of flies	Flies showing manifestation	Penetrance Per cent.	Expressivity Per cent.
Normal food (43 cc)	3,027	727	19	12
Moist food (daily addition of 6 to 7 drops of water in each bottle)	690	162	22	6
Dry food (20 per cent. less water)	3,210	1,913	60	42
Small amount of food (13 cc)	4,258	2,930	68	53
Small vials, small amount of food (10 cc)	721	683	95	75
Old food (20 days old, previously used by one generation of flies)	942	714	76	61
Small amount of old food .	383	383	100	100

flies showing the highest degree of manifestation were always easy to recognize as much smaller than the phenotypically normal flies supports this conclusion. Over-populated stocks showed an increase (of 14 per cent.) in the penetrance, as was to be expected from the fact that lack of food affects the degree of manifestation. It could be shown, finally, that the percentage of flies exhibiting manifestation increases toward the end of the hatching-period: the flies hatching last show the most extreme degree of abnormality (Fig. 2).

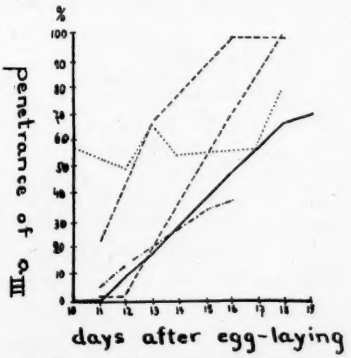

FIG. 2. Diagram of the penetrance of a-III during the single days of the hatching-period. ——— = normal food, ... = dry food, –.–.–.– = moist food, – – – – – – = small amount of food.

Comparing these results with Morgan's studies on "Abnormal Abdomen," we find a striking contrast in the conditions which increase the manifestation of these two abnormal abdomen mutations. They seem to be equal phenotypically but are different

genetically and need just the opposite conditions for their realization. The manifestation of Morgan's "Abnormal Abdomen" depends greatly on the moistness of the food, while our "abnormal abdomen" requires the opposite condition: lack of food or dry food. (Dry food renders the creeping through the food more difficult for the larvae and thus effects the same condition as lack of food). Morgan's "Abnormal Abdomen" shows the highest degree of manifestation in the flies which hatch first during the hatching-period, while the manifestation of a-III increases toward the end of the hatching-period.

One may conclude from this result that two varying characteristics, which are phenotypically similar but differ genetically, are not dependent on the same environmental conditions for their manifestation. The genetical difference may, in fact, require different, even opposite, environmental conditions for the manifestation of each gene.

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